PART I : THE ABORTIVE INFECTION OF BACTERIOPHAGE ØX174 AT LOW TEMPERATURES

PART II : THE EARLY STAGES IN THE PROCESS OF INFECTION BY BACTERIOPHAGE ØX174

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ABSTRACT

Part I.

The infection of E. coli by ØX174 at 15°C is abortive; the cells are killed by the infection but neither mature phage nor SS (single-stranded) DNA are synthesized. Parental RF (replicative form) is formed and subsequently replicated at 15°C. The RF made at 15°C shows normal infectivity and full competence to act as precursor to progeny SS DNA after an increase in temperature to 37°C. The investigations suggest that all of the proteins required for SS DNA synthesis and phage maturation are present in the abortive infection at 15°C.

Three possible causes are suggested for the abortive infection at 15°C: (a) A virus-coded protein whose role is essential to the infection is made at 15°C and assumes its native conformation, but its rate of activity is too low at this temperature to sustain the infection process. (b) Virus maturation may involve the formation of a DNA-protein complex and conformational changes which have an energy threshold infrequently reached at 15°C. (c) A host-coded protein present in uninfected cells, and whose activity is essential to the infection at all temperatures, but not to the host at 15°C, is inactive at 15°C. An hypothesis of this type is offered which proposes that the temperature-limiting factor in SS DNA synthesis in vivo may reflect a temperature-dependent property of the host DNA polymerase.

Part II

Three distinct stages are demonstrated in the process whereby ØX174 invades its host:

(1) Attachment: The phage attach to the cell in a manner that does not irreversibly alter the phage particle and which exhibits "single-hit" kinetics. The total charge on the phage particle is demonstrated to be important in determining the rate at which stable attachment is effected. The proteins specified by ØX cistrons II, III and VII play roles, which may be indirect, in the attachment reaction.

(2) Eclipse: The attached phage undergo a conformational change. Some of the altered phage particles spontaneously detach from the cell (in a non-infective form) while the remainder are more tightly bound to the cell. The altered phage particles detached (spontaneously or chemically) from such complexes have at least 40% of their DNA extruded from the phage coat. It is proposed that this particle is, or derives from, a direct intermediate in the penetration of the viral DNA.

The kinetics for the eclipse of attached phage particles are first-order with respect to phage concentration and biphasic; about 85% of the phage eclipse at one rate ($k = 0.86\ min^{-1}$) and the remainder do so at a distinctly lesser rate ($k = 0.21\ min^{-1}$).

The eclipse event is very temperature-dependent and has the relatively high Arrhenius activation energy of 36.6 kcal/mole, indicating

the cooperative nature of the process. The temperature threshold for eclipse is 17 to 18°C.

At present no specific ØX cistron is identified as affecting the eclipse process.

(3) DNA penetration: A fraction of the attached, eclipsed phage particles corresponding in number to the plaque-forming units complete DNA penetration. The penetrated DNA is found in the cell as RF, and the empty phage protein coat remains firmly attached to the exterior of the cell. This step is inhibited by prior irradiation of the phage with relatively high doses of UV light and is insensitive to the presence of KCN and NaN_3. Temporally excluded superinfecting phages do not achieve DNA penetration.

Both eclipsed phage particles and empty phage protein coats may be dissociated from infected cells; some of their properties are described.

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GENERAL INTRODUCTION

The system of study has been the bacteriophage ØX174 and its host E. coli C. For a complete review of the system the reader should consult the articles by Ray (1968), Sinsheimer (1968a), and Hutchison (1969).

Virus structure. ØX174 is a small spherical virus (Hall, Maclean & Tessman, 1959) of molecular weight 6.2×10^6 daltons of which 25% represents DNA and 75% represents coat protein (Sinsheimer, 1959a). Each phage particle contains one circular molecule of single-stranded (SS) DNA (Sinsheimer, 1959b; Fiers & Sinsheimer, 1962). Purification of the phage from cell lysates reveals the presence of a defective virus particle (Sinsheimer, 1959a). In many virus systems this type of defective particle has been called "top component" since upon CsCl density gradient centrifugation at a mean density close to the buoyant density of the intact virion, this species distributes in the gradient as a band between the virus band and the meniscus. ØX top component is a very heterogeneous species composed of a complete viral protein coat and a variable portion of the viral DNA (Eigner, Stouthamer, Van der Sluys & Cohen, 1963). The particles contain from 0 to 35% of the DNA complement present in intact phage; the distribution has a maximum value at about 17%. This DNA has the form of linear SS DNA molecules with a number average molecular weight of 250,000 daltons (Eigner et al, 1963, Eigner & Doty, 1965). Each 70 S particle therefore contains a single linear

fragment of the viral DNA and while in the 70 S particle it is resistant to digestion by DNase (Sinsheimer, 1959a). As well as being heterogeneous in composition, light scattering studies reveal that ØX top component is also heterogeneous in size and shape (Sinsheimer, 1959a). The particles of ØX top component are not infectious and are believed to derive mostly from abortive infections of intact phage with cell debris in the lysates (Bleichrodt & Knijnenburg, 1969).

The structure of the intact phage is quite sophisticated, considering the limited information content of the virus (about 1,850 codons). In the electron microscope, it exhibits icosahedral symmetry and displays a characteristic "knobby" appearance (Tromans & Horne, 1961). These knobs or spikes in the phage coat appear to be protein appendages to a more simple capsid structure that clothes the viral DNA (Edgell, Hutchison & Sinsheimer, 1969). There are 12 spikes per phage particle, each assigned to one of the 12 vertices of the icosahedron and situated at a five-fold rotation axis (Tromans & Horne, 1961). Incubation of the phage in 4.0 M urea for 3 hr at 30°C dissociates the spikes from the capsid and the subviral components can be separated by sucrose gradient centrifugation (Edgell et al., 1969). Acrylamide gel electrophoresis of these components after further disruption reveals that whole phage contain at least four distinct polypeptide species (Edgell et al., 1969; Burgess & Denhardt, personal communication). The phage capsid is comprised of only one of these species while the spikes account for the remaining

three polypeptides.

ØX DNA The DNA in the intact virion is a single-stranded, covalently closed circular molecule of molecular weight 1.7×10^6 daltons (Sinsheimer, 1959b; Fiers & Sinsheimer, 1962). Under the normal conditions of infection neither parental nor progeny SS DNA is found free in the infected cells (Sinsheimer, Starman, Nagler & Guthrie, 1962; Knippers, Salivar, Newbold & Sinsheimer, 1969). In infected cells ØX DNA accumulates as the SS form in virus particles or as the double-stranded replicative form (RF) (Sinsheimer et al., 1962).

ØX RF DNA has a molecular weight of 3.5×10^6 daltons and is also a circular molecule (Kleinschmidt, Burton & Sinsheimer, 1963). The circular RF DNA is isolated from the cell in two distinct forms; component 1 --- a supercoiled, completely covalently closed, double-stranded circle, and component II --- a non-supercoiled form, containing one or more single-stranded breaks (Burton & Sinsheimer, 1965).

Circular, non-catenated dimers, trimers and higher multimers of RF are also present in infected cells as minor molecular species (Goulian & Kornberg, 1967, Rush, Kleinschmidt, Hellmann & Warner, 1967). It remains yet to be proven that the RF multimer plays some role of consequence in the infective process and is not an artifact of the dying cell; the RF dimer is implicated as a possible intermediate in genetic recombination (Rush & Warner, 1968).

All of these forms of ØX DNA are infectious to bacterial spheroplasts (Guthrie & Sinsheimer, 1963). Circular SS DNA is usually found to be about 20 times as infectious as RF DNA (Burton & Sinsheimer, 1965). The three RF species, I, II and the circular dimer exhibit comparable infectivity in the spheroplast assay (Rush & Warner, 1967). The progeny phage produced in these spheroplast infections all contain circular SS DNA of molecular weight 1.7×10^6 daltons.

Infective Process The initial stages of the process of infection are the subject of part II of this thesis.

(a) Attachment The infection is initiated by the direct attachment of phage to the bacterial cell wall (Stouthamer, Daems & Eigner, 1963), the bacterial receptor site is a phenol insoluble lipopolysaccharide material (J. A. Cameron, personal communication). E. coli C cells have a large number of phage receptor sites, of the order of 50,000 per cell. ØX-resistant cell strains are all characterized by an inability to stably absorb the phage. The character of the receptor material in the C/ØX strains is not yet documented.

Phage attachment per se causes no detectable harm to the host cells nor involves any irreversible structural change to the virus. Mutations in at least three ØX genes (two spike genes .... II and III, the capsid gene ... VII) can alter the attachment properties of the phage.

(b) Eclipse Following phage attachment is the very temperature dependent process designated eclipse. The event involves an irreversible conformational change in the phage particle but no change in its composition. The altered phage particles can be recovered from phage-cell complexes by elution with 0.05M sodium tetraborate, 6mM EDTA. The eclipsed phage particles are no longer infectious to whole cells, but are infectious to spheroplasts. The conformation of the eclipsed phage particle is that of the phage protein coat with the viral DNA partially protruded. The temperature threshold for phage eclipse is 17 to 18°C. A very similar, perhaps identical, eclipse of the phage is found upon interaction with the isolated lipopolysaccharide receptor material (J. A. Cameron, personal communication).

Mutation of the virus can alter the eclipse event (Dowell, 1967). The cold-sensitive mutant cs 70 does not go into eclipse at 25°C, although it attaches stably to the cell. The assignment of this mutant to one of the ØX cistrons remains uncertain at present.

(c) DNA penetration The plaque-forming units in a phage stock (most often the minor fraction), after a successful eclipse, proceed to introduce their DNA into the host cell. This event requires some function or favorable condition of the host cell. Starved cells maintained in the starvation medium do not permit the viral DNA of eclipsed phage to penetrate (Knippers, Salivar et al., 1969). However, immediately upon the

addition of nutrients DNA penetration ensues. Under the normal infection conditions, DNA penetration results in the generation of an empty phage protein coat still firmly attached to the exterior of the cell and the penetrated viral DNA, found as a so-called parental RF molecule. This type of penetration is not inhibited by chloramphenicol, potassium cyanide or sodium azide (Knippers, Salivar et al., 1969). Temporally excluded, superinfecting phage particles do not undergo DNA penetration (Knippers, Salivar et al., 1969); their fate is to form eclipsed phage particles.

If ØX infection is carried out under conditions that block DNA synthesis then a different kind of DNA penetration event is found (Knippers, Salivar et al., 1969). The empty phage coat is produced as before but now free SS DNA is found associated with the cell. This DNA is presumed to be truly inside the bacterial cytoplasm, although this remains to be documented. The penetrated SS DNA is attached to the cell membrane and also contains a high proportion of linear molecules (Heath, personal communication).

A rather strange mutant of the phage, tss6, is known in which the viral DNA does not penetrate the host under the restrictive condition (Dalgarno & Sinsheimer, 1968). This mutant, assigned to ØX cistron III, does go into eclipse, although there is an indication that the eclipse may be aberrant.

(d) Parental RF synthesis. The conversion of the infecting SS DNA to

the parental RF is the first stage of ØX DNA replication. No intermediates in the process have yet been isolated. Since parental RF is also found associated with the bacterial membrane (Knippers & Sinsheimer, 1968), this reaction may be entirely accomplished in vivo on the membrane.

The reaction is effected by enzymes that preexist in the host prior to phage infection (Sinsheimer et al., 1962). Bacterial mutants that are temperature-sensitive in their capacity to replicate DNA (Bonhoeffer, 1966) are unable to synthesize the parental RF at the restrictive temperature (Knippers, unpublished experiments; Steinberg & Denhardt, 1968). The mutants of this type although unable to replicate DNA at the restrictive temperature are competent to undertake UV-induced "repair replication" to bacterial DNA at that temperature (Couch & Hanawalt, 1967), indicating that the synthesis of parental RF in vivo is not simply a special case of host directed repair replication, as has been implied (Sinsheimer 1968b).

(e) The essential bacterial site. At this stage of the infection the parental RF associates with a particular site on the bacterial membrane. It may well be that the viral DNA never becomes dissociated from the bacterial membrane from the time of DNA penetration, throughout the course of parental RF synthesis and of association with the essential bacterial site (Yarus & Sinsheimer, 1967). Furthermore, locating this particular

site may not in fact involve the parental RF moving around inside the cell at all but rather be a case of the site finding the RF molecule and fixing it to the cell membrane. However, the nature of this site remains obscure. Its structure is presumed to be specified by host genes. Many lines of evidence suggest that the parental RF must associate with such a functional site in order that transcription and replication of the parental RF occur (for detailed review, see the article by Sinsheimer (1968a)). The data suggest that the number of such functional sites in the cell is limited and in previously starved cells is most often one. The effect of such a limited number of essential sites is to severely restrict the number of parental RF molecules, and hence phage, that can participate in the infection of one cell. The DNA of the non-participating phage penetrates the cell and forms parental RF. At moderately high multiplicities of infection these RF molecules are also membrane bound (Knippers & Sinsheimer, 1968a), perhaps to nonfunctional sites (Salivar & Sinsheimer, 1969). At very high multiplicities, some parental RF molecules are found not to be associated with cell membrane but are free in the bacterial cytoplasm (Knippers, Salivar, et al., 1969; Salivar & Sinsheimer, 1969).

At this stage of the infection, prior to the onset of any transcription or replication of the parental RF it is not known in what form the RF exists, i.e. component I, II or both.

Conditional lethal mutants of ØX cistron VI are blocked at this stage of DNA replication in the restrictive infection; neither replication of the parental RF nor synthesis of progeny SS DNA occurs (Lindqvist & Sinsheimer, 1967b). This result indicates that transcription of viral genes and viral specific protein synthesis are now required for productive infection to proceed. However, this does not imply that ØX cistron VI need be the first viral gene to be transcribed or translated.

(f) Transcription and translation. The molecular details of the synthesis of ØX mRNA are also obscure. It now seems clear, however, that at no stage of the infection (even at very early times) is ØX mRNA found that bears sequence complementarity to the viral DNA (+strand), i.e. all transcription occurs from the complementary (-) strand of the RF (Hayashi, Hayashi & Spiegelman, 1963; Sedat, personal communication). The ØX mRNA purified from E. coli RNA by chromatography on benzoylated-naphthoylated-DEAE-cellulose (BNC) columns is very polydisperse in size with a mean molecular weight about 0.1×10^6 daltons (Sedat, personal communication). Messengers of size comparable to, and also significantly smaller than ØX DNA exist; it is possible that mRNA molecules are synthesized that are significantly larger than ØX DNA. It seems reasonable at present to assume that transcription generates at least some polycistronic mRNA molecules of information content equal to the complete ØX genome or smaller. The existence of polar mutants (Hutchison, 1969)

provides genetic evidence for polycistronic transcription.

No evidence exists to suggest that a phage-coded RNA polymerase is made in ØX-infected cells. It seems probable that the host RNA polymerase effects the transcription of the viral genes throughout the infection. Certainly the initial transcription event of the parental RF is accomplished by this enzyme (although, conceivably, a phage specific RNA polymerase could be brought into the cell from the infecting phage particle). However, it is recognized that the E. coli RNA polymerase is capable of producing functional ØX mRNA in vitro (Gelfand & Hayashi, 1969).

It also appears that regulation by unknown mechanisms, occurs at either the transcription or translation levels, or at both. In ØX-infected cells the virus coded proteins are not found to be present in equimolar amounts. For example, the phage capsid protein is present in large amount whereas the gene VI protein is a relatively minor protein species (Mayol, personal communication). More evidence to support this suggestion comes from the abortive infection of the rep^- bacterial host (Denhardt, Dressler & Hathaway, 1967). In this mutant eclipse is normal and the parental RF is formed. However, RF replication, cell lysis and cell death (hence also the inhibition of host DNA synthesis) do not occur. It has been postulated that these particular viral genes may not be transcribed, although no analysis of the ØX mRNA in these infected

cells has been made. However, ØX cistron VI protein is present in these cells and therefore gene VI must be transcribed (Levine & Sinsheimer, 1969b). It is conceivable that the rep^+ gene specifies the essential bacterial site and that mutation to rep^- restricts both replication and transcription of parental RF.

Translation of ØX mRNA into viral proteins is assumed to utilize the host apparatus for protein synthesis without any modification. However, no data are available to confirm or negate the assumption. The in vitro synthesis of ØX proteins is accomplished using the translational components obtained from uninfected cells (Bryan, Sugiura & Hayashi, 1969). Nevertheless, in an attempt to explain the specific and non-random base composition (high thymine content) characteristic of all SS DNA viruses, it has been proposed that virus infection alters the translational machinery of the host, perhaps by a limitation on wobble (Denhardt & Marvin, 1969).

ØX infection does not detectably impair the capacity of the host for transcription or translation of bacterial genes. Total RNA and protein synthesis in E. coli appear unaffected by ØX infection (Lindqvist & Sinsheimer, 1967a). Also β-galactosidase is essentially fully inducible in infected cells at the stage when progeny phage are being matured most rapidly (Newbold, unpublished experiments). However, it is noted that replication of RNA phage is inhibited by subsequent superinfection with

ØX174, even after considerable delay (Huppert, Blum-Emerique & Breugnon, 1967); the details and mechanism of this phenomenon are yet to be explored, but it could reflect phage directed modification to the translational machinery of the host.

(g) RF replication In the presence of ØX cistron VI protein and at least two host-coded proteins, viz those coded by the rep^+ allele (Denhardt, Dressler & Hathaway, 1967) and the wild type allele of the Bonhoeffer mutation (Bonhoeffer, 1966) semiconservative replication of the parental RF occurs (Denhardt & Sinsheimer, 1965). Only the parental RF molecules at functional sites replicate (Stone, 1967). This replication is such that the input viral DNA strand always remains associated with the site, exchanging partners at each round of replication. The replicating parental RF molecules are found in the membrane fractions of a cell extract while the replication products, the daughter RF molecules, are not bound to the membrane and are mostly free in the cytoplasm of the cell as RF I molecules (Knippers & Sinsheimer, 1968a).

Intermediates in the semiconservative replication of parental RF have been identified and partially characterized (Knippers, Whalley & Sinsheimer, 1969). As a prelude to replication the parental RF assumes the form of an RF II molecule. The viral (+) strand is an intact ring while the complementary (—) strand contains one break; the specificity of this SS break in the – strand is yet to be examined. Replication

involves the addition of nucleotides to the linear - strands, utilizing the circular + strands as template, so as to produce - strands of greater than viral length. Analysis of pulse labeled parental RF also reveals that the newly synthesized + strands appear as relatively small pieces; the current working hypothesis assumes that the newly synthesized + strand pieces utilize the emerging old part of the complementary strand as template. The mechanism whereby the nascent double-stranded DNA tail maintains or reassumes a circular form remains unknown.

Temperature shift-up experiments with ts mutants in ØX cistron VI suggest that the continued synthesis of cistron VI protein is required for the sustained replication of RF (Levine & Sinsheimer, 1969a). The role of the cistron VI protein in RF replication is also not known. Other proteins, probably mostly specified by the host, must be involved in the replication of RF DNA. Clearly enzyme activities of the DNA polymerase, DNA ligase and endonuclease types are required.

Evidence exists that other host-coded enzymes can act upon RF at this stage of the infection. UV irradiated RF is subject to the dark repair mechanism of the cell (Yarus & Sinsheimer, 1964) and it is conceivable that some of these enzymes act upon RF molecules in the course of normal infection. Also it seems probable that genetic recombination for ØX occurs at the level of RF DNA. Both normal and UV-stimulated ØX recombination are inhibited in the "reckless" type of recombination

deficient (rec^-) cells (Hutchison & Newbold, unpublished experiments).

(h) Synthesis of SS DNA and mature phage. The onset of progeny SS DNA synthesis in infected cells marks an interesting stage in the infection. Within a short interval, and perhaps simultaneously, the following phenomena are observed:-

(i) Cessation of net RF synthesis.

(ii) Cessation of net host DNA synthesis (Lindqvist & Sinsheimer, 1967a).

(iii) Onset of progeny SS DNA synthesis.

(iv) Marked increase in the overall rate of DNA synthesis.

(v) The establishment of temporal exclusion for superinfecting ØX phage (Hutchison & Sinsheimer, 1966); the DNA of the superinfecting phage remains as SS DNA and does not penetrate the cell (Knippers, Salivar, et al., 1969).

Quite possibly all five observations are spawned by a single event in each infected cell. The virus induced inhibition of host DNA synthesis and the establishment of temporal exclusion both require protein synthesis, i.e., neither phenomenon is observed in the presence of chloramphenicol.

No mutants of the phage exist that can penetrate their DNA but not effect the 'shut-off' of host DNA synthesis. The inhibition of host DNA synthesis must not occur in the abortive infection of the rep^-

mutant host since the cells are not killed by the infection (Denhardt et al., 1967). This result would be accounted for if the 'shut off' is effected by a virus-coded, chloramphenicol sensitive protein that is not made in rep⁻ infected cells.

Although net RF synthesis ceases in infected cells, pulse label experiments indicate that RF is synthesized throughout the duration of SS DNA synthesis; these late labeled RF molecules, labeled in their + strands, are precursors to progeny SS DNA (Lindqvist & Sinsheimer, 1968; Sinsheimer, Knippers & Komano, 1968). The pool of cytoplasmic RF I molecules that accumulate during the semiconservative replication of the parental RF are the precursors to SS DNA synthesis. The immediate RF precursor is an RF II molecule of a specific character. This RF II molecule is composed of an intact circular complementary strand and a viral strand containing one nick. The nick is considered to be unique from an analysis of the nucleosides that immediately flank it (Knippers, Razin, Davis & Sinsheimer, 1969); deoxyguanosine is on the 3'-terminus and deoxycytidine on the (non-phosphorylated) 5'-terminus.

SS DNA synthesis involves the addition of nucleotides to the 3'-end of the viral strand with the concomitant displacement of the 5'-end from its complementary strand template. The displaced 5'-end emerges as a non-hydrogen-bonded SS tail from a double-stranded circular RF

molecule. Such intermediates in SS DNA synthesis have been isolated and further shown to be associated with viral coat protein (Knippers, Razin, et al., 1969). The maximum length of the viral strand in the intermediate molecule is twice the length of the viral DNA. Analysis of the 5'-end of the viral strand in the intermediate molecule demonstrates that the terminal nucleoside is no longer deoxycytidine, but rather deoxyguanosine. The mechanism whereby the emergent linear viral strand is excised from the intermediate molecule, circularized and matured into virus particles is not understood.

SS DNA synthesis and progeny phage-maturation are interdependent processes (Sinsheimer, 1968a). As mentioned previously no pool of free progeny SS DNA accumulates in infected cells. The association of viral coat protein with the DNA molecule that is an intermediate in SS DNA synthesis suggests that the maturation of viral DNA into virions begins very early during SS DNA synthesis ..... most probably as soon as the tail of SS DNA emerges from the replicative complex. It seems probable that the viral coat proteins interact with specific nucleotide sequences present in the viral DNA rather than possessing special affinity for SS DNA per se. The latter possibility might permit isolated complementary strands, were they to arise in the infected cell, to be incorporated into phage-like particles. However, an analysis of the progeny phage released from bacteria mixedly infected with

both ØX174 and the filamentous SS DNA phage fd demonstrated that the maturation of fd DNA into ØX protein coats (and also the maturation of ØX DNA into fd protein coats) was an extremely rare event (Knippers & Hoffman - Berling, 1966)

Mutants in five phage cistrons permit essentially normal RF replication but prevent both SS DNA synthesis and maturation. Three of the five cistrons, III, IV and VII, specify structural proteins present in the mature virion; the two remaining cistrons, V and VIII specify unidentified functions. Genes V and VIII may well encode for proteins involved in SS DNA synthesis such as a specific endonuclease, a DNA polymerase or ligase. Such proteins are expected to be present in catalytic quantities in the infected cell, however, cistron V protein, assuming that it has an average amino acid composition, appears to be present in infected cells in stoichiometric amounts ..... more abundant than some structural proteins (Burgess & Denhardt, personal communication). The cistron VIII protein is not identified yet which perhaps indicates that it is present only in catalytic amounts. Cistron VIII is itself defined by only one mutational site (Funk, personal communication); the mutation generates the ochre (nonsense) triplet. The low efficiency of suppressor strains to suppress the ochre mutation has resulted in the surmise that it is the normal chain termination codon in protein synthesis; efficient suppression of chain termination would

presumably be lethal. Hence it has been further conjectured that ochre mutants would only be detected in enzymatic proteins and that they would be lethal in essential structural proteins. It seems probable that cistron VIII specifies an enzyme involved in SS DNA synthesis or phage maturation. Part I of this thesis emphasizes the interdependence of SS DNA synthesis and maturation. Both processes are demonstrated to be acutely cold-sensitive and to have very similar, perhaps identical, temperature thresholds for function.

It is easy to understand how the inability to synthesize SS DNA blocks phage maturation. But how the inability to make functional coat protein imposes a block to SS DNA synthesis, is not so apparent. In light of the mechanism of SS DNA synthesis previously discussed it might be inferred that the lack of functional coat protein to properly interact with the replicating DNA complex might result in either the continued replication of RF (which is not observed) or the appearance of aberrant RF forms (which have not yet been sought).

ØX cistron VI protein which is required for the continued synthesis of RF does not play an essential role in SS DNA synthesis (Levine & Sinsheimer, 1969). Mutant host cells (Bonhoeffer, 1966) temperature-sensitive in their capacity to synthesize DNA are not able to support the synthesis of progeny SS DNA (Steinberg & Denhardt, 1968). Also the rep^+ gene product may participate in an essential way in SS DNA

synthesis ..... although at present this possibility cannot be assayed.

No intermediate stages in phage maturation beyond the replicating DNA intermediate - viral coat protein complex are known. However, three distinct subviral particles are matured in the restrictive infection produced by conditional lethal mutants of ØX cistron II which could be direct intermediates in the normal process of virus assembly. A preliminary characterization of the three components is available for the case of ts 4 infection at 40°C. The three species are separated by sucrose gradient centrifugation as 107S, 71S and 21S components identified by their serum blocking power (Krane, 1966). The 107S component has the appearance of intact phage in the electron microscope (i.e. spherical 'knobby' virion) but it is not infectious to cells. The defective particle contains an infectious SS DNA molecule but it lacks one specific spike protein (Edgell et al., 1969) and is unable to attach to cells. The phenotype of the ts 4 mutation suggests that the incorporation of the gene II protein into phage may represent the terminal step of virus assembly. It is not known if the 110S defective ts 4 particle is converted to an infectious 114S virion upon shifting ts 4-infected cells from 40° to 30°C.

(i) Lysis and phage release. Normal ØX infection terminates with the virus-induced lysis of the host cells and the release of the progeny phage in a brief burst (Hutchison & Sinsheimer, 1963). Considerable

asynchrony exists in a population of so-called synchronously infected cells for the time of the burst. One ØX cistron exhibits a marked effect upon the lysis phenomenon. Mutants in cistron I do not lyse the host and phage production continues giving greatly increased virus yields (Hutchison & Sinsheimer, 1966). The role of the gene I protein remains obscure. No lysozyme activity is yet recognized in ØX-infected cells. Conceivably the cistron I protein might interfere with cell wall synthesis (Hutchison & Sinsheimer, 1966).

Electron micrographs of thin sections of ØX-infected cells indicate that cell lysis involves a progressive damage to the cell wall (Bradley, 1968). The damaged cells, containing mature virions, appear somewhat swollen usually with a large bulge in the equatorial regions of the cell. The plasma membrane retracts from the poles of the cell leaving a distinct gap between the membrane and the cell wall. The cells appear to be converted to a spheroplast form. In the 'bulge region' breaks appear in the cell wall which later enlarge as the walls seem to curl up. Later still, the plasma membrane in the vicinity of the bulge ruptures, releasing the bacterial cytoplasm and the intracellular phage.

ØX cistrons At present eight cistrons have been defined for the ØX genome: -

I Lysis.

II Spike protein; involved in phage attachment to cell.

III Spike protein; the serum blocking antigen; possibly directly involved in phage attachment, eclipse and DNA penetration.

IV This protein has not been identified yet. It is inferred to be a structural protein (perhaps the 'unclaimed' spike protein) since several mutations in this gene alter the thermosensitivity of the virion (Hutchison, 1969). The cistron also harbors three distinct ochre mutations (Funk, personal communication), and as previously discussed this might indicate that the gene IV protein has an enzymatic function.

V This is clearly not a structural protein of the phage; perhaps involved in SS DNA synthesis and phage maturation.

VI Required for RF replication

VII Capsid protein; involved, perhaps indirectly, in phage attachment to cell; determines host range.

VIII The protein is not yet identified; probably involved in SS DNA synthesis or phage maturation.

Proteins Six distinct phage coded polypeptides have been resolved by acrylamide gel electrophoresis in the presence of SDS (Burgess & Denhardt, personal communication). Five of these proteins have been assigned to their structural genes, viz. I, II, III, V and VII; one

polypeptide, known to be a component of the phage spikes, remains unassigned to a structural gene. The electrophoresis analysis provides some data on the molecular weights of these proteins: -

cistron I protein	5,000 to 15,000 daltons
cistron II protein	40,000 daltons
cistron III protein	23,000 daltons
cistron V protein	15,000 daltons
cistron VII protein	48,000 daltons
the unassigned spike protein,	5,000 to 15,000 daltons.
Total -	136,000 to 156,000 daltons.

The cistron VI protein has been partially purified and characterized (Levine & Sinsheimer, 1968) and its molecular weight is estimated to be 10,000 to 20,000 daltons. The known proteins then, comprise 146,000 to 176,000 daltons of protein. If it is assumed that ØX cistron VIII specifies a protein of molecular weight 20,000 daltons, then it can be concluded that ØX DNA encodes for 166,000 to 196,000 daltons of protein. This amount of protein corresponds to some 1,400 to 1,660 amino acid residues (using a molecular weight of 118 daltons for the "average" amino acid). ØX DNA corresponds to about 1,850 triplets. If it is assumed that ØX employs the non-overlapping triplet code and that all of the ØX triplets are translated into protein, then these rough calculations indicate that 190 to 450 triplets are unaccounted

for. This amount of information could define from one to 20 genes - the E. coli DNA polymerase is a single polypeptide chain of about 850 amino acid residues (Jovin, Englund & Bertsch, 1969) while the A chain of insulin contains only 21 amino acids (Sanger & Thompson, 1953) - but most probably it corresponds to only one or two as yet undefined genes.

Postscript. It has already been discussed that several phage-induced functions could be attributed to the unidentified gene(s) eg., the chloramphenicol sensitive protein that inhibits host DNA synthesis, and the specific endonucleases and ligase required for SS DNA synthesis. However, the unique feature of ØX174 and the major reason for its great interest to molecular biologists has been the single-stranded nature of its DNA. That SS DNA is not synthesized in the presence of chloramphenicol has lead to the speculation of a phage-coded DNA polymerase for SS DNA synthesis (Sinsheimer, 1961). Evidence has been presented to show that in ØX-infected cells there is a phage induced stimulation of the DNA polymerase activity of from two - to five-fold (Huh & Helleiner, 1968). The phage induced polymerase activity is claimed to have different ionic conditions for optimal activity than the enzyme from uninfected cells; the enzyme is reported to be purified 1,000-fold. Similar studies have been made but generating

different results by Grunau and Daniel (1968). These authors find that sometimes polymerase activity is enhanced in extracts of infected cells but that it is more readily attributable to artifacts arising in the assay system caused by other components in the crude enzyme preparations; purification of the polymerase activity from the crude extracts results in loss of both quantitative and qualitative differences between "infected" and "uninfected" enzyme preparations.

To date no data exist that demand the concept of a ØX-coded DNA polymerase for SS DNA synthesis. Indeed the investigation of the synthesis of ØX SS DNA by infected cell extracts in vitro suggests otherwise (Denhardt, 1968). In this system the small amount of ØX SS DNA synthesis that is observed is found to be sensitive to antiserum directed against the purified DNA polymerase of E. coli, although less sensitive than the 'total' DNA synthesis of this system. The result suggests that the synthesis of ØX SS DNA is effected either by the unmodified host enzyme or an enzyme that bears homology to it. An homologous enzyme could be produced by an association of the host enzyme with a phage-coded protein, however, an homologous phage-coded polymerase of molecular weight comparable to the host enzyme (100,000 daltons) would seem to be excluded.

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PART I

THE ABORTIVE INFECTION OF BACTERIOPHAGE ØX174 AT LOW TEMPERATURES

I.1 Introduction

The rates of growth of bacteriophage and bacteria are both inhibited at low temperatures, the lower the temperature the slower the growth rate. This property is widely exploited in microbiological research as a very convenient method of arresting the growth of cultures by simply setting them into an icebath. However, the minimal growth temperature for bacteria is usually not $0^{o}C$ and presents somewhat of an enigma that was clearly stated by Foter and Rahn. "Since fermentation and growth are chemical reactions (as temperature is decreased) they should continue though at a greatly reduced speed until the medium freezes solid . This is certainly not always the case with the growth of bacteria; most of them completely cease to grow at temperatures five to ten or more degrees above the freezing point " (Foter & Rahn, 1936). It has been proposed that a major reason for the existence of a minimal temperature of bacterial growth is that the regulation of the synthesis of enzymes is deranged at low temperature (Ng, Ingraham & Marr, 1962). In general, control mechanisms are found to be more rigorous at low temperature; certain enzymes cannot be induced and the rate of synthesis of others decreases dramatically. This altered physiology has been documented for the growth of E. coli at low temperatures and it will be discussed further later in this introduction.

It is characteristic of all viruses that they utilize the ribosomes

of, and derive their metabolic energy from, the host cell. To this extent all bacteriophages are dependent on their hosts. Some phages require little else and their replication is relatively autonomous (eg. T1, T2, T4, T5, T7, P1 and the RNA phages); other phages (like , ØX174 and M13) are more dependent upon their hosts and have adapted their replication schemes so as to utilize other host-coded enzymes and bacterial replication sites (Sinsheimer, 1968). Whatever the degree of autonomy enjoyed by a bacteriophage its minimal temperature of growth might be imposed by the altered physiology of its host at low temperatures. Consider a bacteriophage system for which the minimal temperature of growth is T_a and that for the host bacterium is T_b. Three possible situations can be envisaged, viz, (i) $T_a = T_b$, (ii) $T_a < T_b$ and (iii) $T_a > T_b$. Case (i) might obtain if the cold-sensitive lesion in the phage infection is imposed by the altered physiology of the host at the low temperature. Cases (ii) and (iii) require more involved explanations. The cold-sensitive lesion in the infection might be a virus-coded protein and its critical temperature might be greater or less than T_b. Alternatively the cold-sensitive lesion could be a host-coded function that is not involved with the restriction of growth of the host at low temperatures, and its critical temperature could in principle also be greater or less than T_b. Few studies have been made of the physiology of phage infections at low

temperatures, nevertheless it seems probable that bacteriophage systems representative of cases (i), (ii) and (iii) do exist. T3 seems to be an example of case (ii) (Doermann, 1948), and the studies reported in part I of this thesis document ØX174 as an example of case (iii).

This introduction will continue by first reviewing the physiology of E. coli at low temperatures. Next the brief literature related to the minimal temperature of coliphage replication will be discussed.

The minimal temperature of growth for E. coli is in the range of 8 to 10°C depending on the complexity of the growth media. For glucose-minimal media the value is 10°C, while for more complex nutrient broth media it is 8°C (Ng, 1963). If the logarithm of the bacterial growth rate is plotted as a function of the reciprocal of the absolute temperature according to the Arrhenius equation, then the slope of this function is directly proportional to the temperature coefficient of the process. In both complex and glucose-minimal media the temperature coefficient for growth of E. coli is constant over a range of temperature (15°C to 37°C in complex media, 20° to 37°C for minimal media). This range has been denoted as 'normal temperature'. At low temperature the temperature coefficient increases progressively and approaches infinity as the temperature approaches the minimum for growth (Ng, 1963).

The first evidence for a derangement of regulatory mechanisms

was the response of growing cultures to a sudden change of temperature (Ng, Ingraham & Marr, 1962). If a change is made within the range of normal temperature the response is immediate. After a change from 25°C or 30°C to 37°C the culture grows exponentially, without lag, at a rate characteristic of the steady-state at 37°C. A change from low to normal temperature results in a transient growth rate before the normal growth rate is attained. After the increase from 10°C to 37°C, the culture grows exponentially but at 0.3 of the rate which is normal at 37°C. The rate of growth immediately following the increase is a function of the initial temperature; however, the attainment of the normal growth rate requires about 2.3 doublings, regardless of the initial temperature of growth.

A transient growth rate also follows a sudden decrease from normal to low temperature. Immediately after the decrease in temperature from 37°C to 10°C, growth stops, after which the growth is more rapid than normal for about one doubling; then the growth rate characteristic of the steady-state at 10°C is assumed (Ng, Ingraham & Marr, 1962).

These studies illustrate that growth at low temperature results in a unique physiological damage to the cell. Growth at low temperature is required before this damage is expressed, and subsequent growth at normal temperature is required to correct the damage. The

kinetics of recovery of the normal growth rate after transfer from low temperature are in agreement with a model based on a process with two sequential steps (Ng, Ingraham & Marr, 1962).

Cells grown in the low temperature range are also characterized by a different fatty acid composition from those grown at normal temperatures. The lipids of E. coli grown at low temperatures have a higher proportion of unsaturated fatty acids than the lipids of cells grown at normal temperatures (Marr & Ingraham, 1962). It has been proposed that the composition of the lipids of microorganisms may set the limits of temperature for growth (Heilbrunn, 1924; Bělehrádek, 1931; Gaughran, 1947; Kates & Baxter, 1962). However, temperature shift-down studies undertaken by Shaw and Ingraham (1965) demonstrated that the adjustment of the fatty acid composition that normally occurs during the lag period that results from shifting E. coli from 37° to 10°C during the exponential growth is not prerequisite for growth at 10°C. Glucose was withheld during the lag period at 10°C and then returned to the medium after the normal lag period with the result that the cells began to grow at 10°C immediately upon the addition of the glucose but with the fatty acid composition of the lipids still charac - teristic of cells grown at normal temperatures. It was concluded that the fatty acid composition of E. coli is not directly related to its minimum growth temperature.

Derangement of the control mechanisms of the cell seemed to be a more likely basis for the physiological damage that occurs during bacterial growth at low temperatures. This would also account for the two-step kinetics mentioned previously; the first step would be the restoration of the normal regulation, and the second step, the correction of enzyme composition. This hypothesis has been strengthened by the finding that such cells exhibit a decreased level of inducibility for at least two inducible enzymes. Tryptophanase is not induced in E. coli grown at 15°C (Ng, 1963); β-galactosidase is induced under these conditions, but to a reduced level (Marr, Ingraham & Squires, 1964).

Mutants which can form tryptophanase at 13°C have been isolated (Gartner & Riley, 1965a) and they are of two types. One group is closely linked to the structural gene for tryptophanase and is characterized by being inducible at 13°C, constitutive above 20°C, and hyperinducible at 37°C; the other group is not closely linked to the structural gene and is constitutive at 13°C (Gartner & Riley, 1965b).

Another system which is regulated by a cytoplasmic repressor is the prophage state of bacteriophage λ (Jacob & Monod, 1961; Ptashne, 1967). The stability of λ lysogens was estimated by measuring the ratio of plaque-forming units to bacteria as a function of temperature of growth. The ratio decreases almost three decimal orders as the temperature of growth is decreased from 37°C to 10°C. Control

experiments showed that this effect of temperature is directly attributable to a decreased frequency of spontaneous induction rather than to readsorption or to a change in burst size. Despite the large effect of temperature on the stability of the lysogen, the frequency of lysogenization is not a function of temperature over the range 20° to 40°C (Ingraham, personal communication).

None of the systems that have been discussed are obligatory for the growth of bacteria. The results indicate a change toward more stringent regulation at low temperature. If the synthesis of some indispensible enzyme were inhibited, as is the synthesis of tryptophanase, one could account for the minimal temperature of growth.

If the regulation of an indispensible enzyme were altered to prevent its synthesis at moderate temperature, the minimal temperature for growth of that mutant would be increased. Mutants of E. coli which have higher than normal minimum temperature of growth have been isolated (O'Donovan, Kearney & Ingraham, 1965). Three general classes of these cold-sensitive mutants which do not grow at temperatures below 20°C (although their parents can grow at 8°C) are recognized. The first class of mutants (K-I) cannot grow below 20°C in either complex or minimal media, but grows at nearly normal rates at 37°C on both types of media. Normal growth rate at 20°C can be conferred on these mutants by infection at low multiplicity with a transducing phage

grown on the parent. The second class of mutants (K-II) fails to grow only in minimal medium at 20°C. These mutants are characterized by their singular response to specific nutrients in minimal medium at 20°C. The third class of mutants (K- III) grows normally in minimal medium at all temperatures with either glucose or glycerol as the carbon source, but does not grow at 20°C with lactose as the carbon source.

One of these cold-sensitive mutants, K-II-27 has been further characterized and shown to require histidine for growth only at low temperatures (O'Donovan & Ingraham, 1965). Enzyme analysis of this mutant revealed that the first enzyme in histidine biosynthesis, phosphoribosyl ATP pyrophosphorylase is 1000-fold more sensitive to feedback inhibition by histidine at 37°C than is the same enzyme in the parent. Moreover, the binding of histidine to the mutant enzyme caused the irreversible inactivation of the enzyme. The analysis suggested that the cold-sensitive mutation affected the regulatory site of the allosteric protein; there was no evidence that the catalytic site of the phosphoribosyl ATP pyrophosphorylase was altered. O'Donovan and Ingraham (1965) also concluded that this same enzyme from the parent bacterium was significantly more sensitive to feedback inhibition at 20°C than at 37°C.

From these studies Ingraham has hypothesized that the eons of selection that adapted E. coli to optimal growth at 37°C also rigidly

fixed the minimum growth temperature. He suggests that many allosteric proteins adapted for optimal function at 37°C may become inactive at low temperatures (8° to 10°C). One or more such indispensible proteins might create a biochemical lesion not relieved by complex nutrient media (Ingraham, personal communication).

The first report of bacteriophage replication at low temperatures is contained in the classic paper by Ellis and Delbruck (1939) concerning the one-step nature of their growth. This phage was isolated by Ellis from Los Angeles sewage but was subsequently lost from the 'scene' (Delbruck, personal communication) - presumably, somewhere it awaits rediscovery. The phage, however, was reported to have an efficiency of plating that was constant in the range 10° to 37°C. Moreover, the one-step growth study at 16.6°C revealed that the length of the latent period was altered by the decreased temperature (180 min at 16.6°C, 30 min at 37°C) whereas the burst size (60 PFU/cell) remained unaltered.

Growth of T3 was found to be restricted in the range 0° to 4°C (Doermann, 1948). Some replication of T3 at 5°C is indicated by the data presented in that report, although it is conceivable that this represents only a maturation at 5°C of phage precursor components synthesized prior to the temperature shift-down. However, growth of T3 at 10°C seems certain. Doermann reports that 60 min at 10°C are

required to mature the number of plaque-forming units matured per min at 30°C. No information is available to date on the nature of the cold-sensitive lesion in T3 infection.

The minimal growth temperature for the T-even phages has not been defined. The one-step growth experiment for T4 has been examined at 15°C (Eder & Edgar, unpublished experiments); the latent period for the infection at 15°C was extended and the burst size significantly reduced.

For bacteriophage ØX174 the minimum temperature for replication is 17° to 18°C. This temperature range marks the threshold for at least two distinct phenomena in the infective process: -

(i) The change in the conformation of the phage protein coat necessary for the phage to "eclipse" and thereafter for the viral DNA to penetrate the cell.

(ii) The intimately related processes of single-stranded DNA synthesis and phage maturation.

The investigation of the abortive infection of ØX174 at low temperatures is presented in section I.2 in the form of a manuscript. A detailed discussion of the results is, of course, included in this manuscript. Some more general discussion is presented in section I.3.

I.2 Results

I.2.1. The Abortive Infection at Low Temperatures

The results in this section have been submitted for publication in the Journal of Molecular Biology. The paper is included in the form in which it was submitted.

Running Title: ØX Infection at Low Temperatures.

SUMMARY

The infection of E. coli by ØX174 at 15°C is abortive; the cells are killed by the infection but neither mature phage nor SS* DNA are synthesized. Parental RF is formed and subsequently replicated at 15°C. The RF made at 15° C shows normal infectivity and full competence to act as precursor to progeny SS DNA after an increase in temperature to 37°C. Some 200 phage equivalents of SBP are also found to have been synthesized during 12 hr of infection at 15°C.

The addition of CAM to 100 μg/ml. to abortively infected cells late in the infection at 15°C does not block the subsequent maturation of infectious virus following an increase in temperature to 37°C. The data suggest that, given an adequate pool of the essential proteins and the precursor RF, the concomitant synthesis of protein is not required for the synthesis of SS DNA or for phage maturation. It is concluded that all of the proteins required for SS DNA synthesis and phage maturation are present in the abortive infection at 15°C.

Temperature "shift-up" experiments (15°C to 41°C) performed with temperature-sensitive mutants of ØX174 and carried out in the presence of CAM as just described demonstrate that the proteins coded by ØX cistrons II, III, IV, V and VII are synthesized at 15°C and probably adopt their native conformation at this temperature. The proteins coded by several ØX strains mutant in cistrons II, III, IV and VII are shown to

be functional at 41°C, when synthesized at 15°C, although they are inactive when synthesized at 41°C. These experiments indicate that the majority of ts mutations impose their restriction during the folding of the nascent polypeptide into the native conformation.

Three possible causes are suggested for the abortive infection at 15°C: (a) A virus-coded protein whose role is essential to the infection is made at 15°C and assumes its native conformation but its rate of activity is too low at this temperature to sustain the infection process. (b) Virus maturation may involve the formation of a DNA-protein complex and conformational changes which have an energy threshold infrequently reached at 15°C. (c) A host-coded protein present in uninfected cells, and whose activity is essential to the infection at all temperatures but not to the host at 15°C, is inactive at 15°C. An hypothesis of this type is offered which proposes that the temperature-limiting factor in SS DNA synthesis in vivo may reflect a temperature-dependent property of the host DNA polymerase.

1. INTRODUCTION

Abortive infections induced by conditional lethal mutations, drugs or radiation treatments of the virus or the host have been used to dissect the process of virus replication in infected cells. The use of low temperatures as a restrictive condition for virus replication is uncommon. Infection with bacteriophage ØX174 is abortive at 15°C. Since the host, E. coli, undergoes exponential growth at 15°C, it must of necessity successfully undertake a wide variety of macromolecular syntheses. Infection of E. coli by phage T4 is essentially normal at 15°C (Eder and Edgar, unpublished experiments); the latent period for the infection at 15°C is of course extended and the final phage yield slightly reduced. Recent studies on the morphogenesis of T4 (Edgar & Lielausis, 1968) would suggest that the in vivo construction of this phage is likely to be a more complex task than for the small icosahedral ØX174. Why then is ØX174 unable to replicate at 15°C, and which essential virus- or host-coded function aborts? Conceivably this phenomenon might be related to the single-stranded nature of ØX DNA.

Although E. coli does exhibit exponential growth at 15°C, the physiology of the host appears to be altered at this temperature. Marr & Ingraham (1962) showed that the lipids of cells grown at low temperatures have a higher proportion of unsaturated fatty acids than the lipids of cells grown at higher temperatures. Further, such cells exhibit a

decreased level of inducibility for at least two inducible enzymes. Tryptophanase is not induced at 15°C (Ng, 1963); β-galactosidase is induced at this temperature, but to a reduced level (Marr, Ingraham & Squires, 1964). The possibility must therefore be entertained that ØX infection aborts at 15°C as a result of the altered physiology of the host at low temperatures.

2. MATERIALS AND METHODS

(a) Escherichia coli strains

(i) C is the usual host of ØX used in this laboratory, BTCC no.122.

(ii) HF4704 is an hcr^- and thymine-requiring strain described by Lindqvist & Sinsheimer (1967a). It is a non-permissive host for ØX amber mutants.

(iii) HF4714 is a multiple auxotrophic strain used as the permissive host for ØX amber mutants.

(b) ØX174 strains

(i) wt is the ØX174 wild type as characterized by Sinsheimer (1959).

(ii) ØXam3 is a lysis-defective amber mutant of ØX174, characterized by Hutchison & Sinsheimer (1966), and assigned to ØX cistron I.

(iii) ØX$ts_{\delta}h$ is an extended host range, temperature-sensitive mutant; its use as a biological marker is described by Hutchison & Sinsheimer (1966).

(iv) ØXam3ts4 is a double mutant, amber in cistron I (lysis) and temperature-sensitive in cistron II (coat protein).

(v) ØXam3ts$_{\gamma}$ is a double mutant, amber in cistron I (lysis) and temperature-sensitive in cistron III (coat protein).

(vi) ØXam3ts79 is a double mutant, amber in cistron I (lysis) and temperature-sensitive in cistron III (coat protein).

(vii) ØXam3ts9 is a double mutant, amber in cistron I (lysis) and temperature-sensitive in cistron IV (coat protein).

(viii) ØXam10 is an amber mutant assigned to cistron V (function unknown). Growth of am10 in HF4714 cells is markedly temperature-sensitive.

(ix) ØXam3ts41D is a double mutant, amber in cistron I (lysis) and temperature-sensitive in cistron VII (coat protein).

All ØX mutant strains, with the exception of ts41D, were isolated by Clyde A. Hutchison, III; ts41D was isolated by Dr. C. Dowell; all ØX double mutant strains were constructed by Clyde A. Hutchison, III.

(c) Media

(i) KC broth contains 10 g Bacto Tryptone (Difco), 5 g KCl and 1.0 ml. 1 M $CaCl_2$ per liter of distilled water.

(ii) SVB (starvation buffer) contains 5 g KCl, 1 g NaCl, 1.14 g Trizma HCl (Sigma), 0.33 g Trizma Base (Sigma), 0.1 g $MgSO_4$ and 1.0 ml. 1 M $CaCl_2$ per liter of distilled water. This is essentially the recipe described by Denhardt & Sinsheimer (1965).

(iii) TPA medium is composed of 8.0 g KCl, 0.5 g NaCl, 1.1 g

NH_4Cl, 0.2 g $MgCl_2$, 6 H_20, 0.23 g KH_2PO_4, 0.8 g sodium pyruvate, 11.44 g Trizma HCl, 3.32 g Trizma Base, 2.7 g of amino acid mixture (special 20 natural amino acid mixture, Nutritional Biochemicals Corporation), 1.0 ml. 1 M $CaCl_2$ and 1.0 ml. 0.16 M Na_2SO_4 per liter of distilled water. The addition of 0.2 ml. of 10% glucose solution to 10 ml. of TPA medium permits full bacterial growth.

(iv) TPA2X medium is composed of 11.0 g KCl, 2.2 g NH_4Cl, 0.4 g $MgCl_2$,6 H_2O, 0.46 g KH_2PO_4, 1.6 g sodium pyruvate, 22.88 g Trizma HCl, 6.64 g Trizma Base, 5.4 g of amino acid mixture, 1.0 ml. 1 M $CaCl_2$ and 2.0 ml. 0.16 M Na_2SO_4 per liter of distilled water.

The mixture of equal volumes of TPA2X and SVB yields a medium with the approximate composition of TPA. For such purposes, 0.4 ml. of 10% glucose solution is added to 10 ml. of TPA2X medium.

(d) Biological assays

(i) The plating procedures, infectious DNA assay, intracellular phage measurements and the preparation of genetically pure mutant phage stocks used here are those described by Hutchison & Sinsheimer (1966).

(ii) Uninfected bacteria are measured by diluting the infected culture into KC broth at 0^oC, and plating for residual colony formers by spreading an aliquot over the surface of an agar-tryptone medium in the presence of anti-ØX serum. The serum inhibits the killing of uninfected bacteria on the plate due to the lysis and release of phage from the

infected cells. 0.25 ml. of serum with a K of 100 min^{-1} is used per plate. The use of the serum is not required when the infected cells are lysis-inhibited, as for am3-infected E. coli C.

(e) $^{32}PD^{15}N$-ØXam3

These phage were prepared with slight modification according to the procedure described by Sinsheimer, Starman, Nagler & Guthrie (1962). The heavy medium was buffered by Trizma HCl and Trizma Base and the salts were reduced to dryness in the presence of D_2O in a flash evaporator. It was found that this procedure resulted in a significant loss of $^{15}NH_4Cl$ from the medium, presumably by sublimation, and so $^{15}NH_4Cl$ was routinely added to the salts-D_2O solution after the flash evaporation step. The D-TGL medium was dispensed into glass ampules, sealed and auto-claved.

E. coli C cells were collected after 10 hr of infection at 37°C by am3 in heavy medium and in the presence of ^{32}P, lysed by lysozyme-EDTA treatment and the phage purified first by CsCl equilibrium density gradient centrifugation and then by velocity sedimentation in a sucrose gradient. In the raw lysate the phage yield was 300 PFU per cell. The purified heavy phage had a buoyant density of 1.46 g/ml. and a specific activity of 3.3×10^{-5} ^{32}P d.p.m. per PFU.

(f) Chemicals

(i) Thymidine-methyl-^3H and thymine-methyl-^3H were purchased

from New England Nuclear Corporation.

(ii) ^{3}H-L-leucine was purchased from Schwarz Bioresearch Inc.

(iii) ^{32}P was purchased from Nuclear Consultants.

(iv) D_2O (99.86% D_2O) and $^{15}NH_4Cl$ (99% ^{15}N) were purchased from BioRad Laboratories.

(v) Mitomycin C was obtained from Nutritional Biochemicals Corporation.

(vi) Chloramphenicol (CAM) was the generous gift of Parke, Davis & Company.

(g) Infection procedures at 15°C

The bacterial cells were grown at 37°C in either KC broth or TPA medium plus glucose, to a concentration of 1 to 2 x 10^8 per ml. The cells were pelleted by centrifugation, washed once with SVB, and then resuspended in SVB (often this last step entailed a concentration of the cells). The cells were then starved in SVB at 37°C for 60 to 90 min. Phage were added, and after 5 to 8 min at 37°C the cultures were set into an ice bath. The cultures were then transferred to a water bath at 15°C and the development of the infection process initiated by the addition of an equal volume of either KC broth or TPA2X medium plus glucose, also at 15°C.

When specific labeling of viral DNA was required, E. coli HF4704 was utilized and was treated during the starvation period at 37°C with mitomycin C (50 μg/ml.) for 10 min (Lindqvist & Sinsheimer, 1967b).

These cells were then pelleted by centrifugation, washed once with SVB, and finally resuspended in fresh SVB and infected with phage.

In the majority of the experiments undertaken to investigate the nature of the abortive infection at low temperatures, the lysis-defective mutant am3 and the non-permissive hosts E. coli C and HF4704 were used to obviate the problems of lysis.

(h) Extraction of DNA from infected cells

The procedure used was that described by Lindqvist & Sinsheimer (1967a).

(i) ^{32}P SS DNA for use as a marker

The procedure used was essentially that described by Lindqvist & Sinsheimer (1967a); the phage, however, were first purified by velocity sedimentation in a sucrose gradient and then by CaCl equilibrium density gradient centrifugation.

(j) Centrifugation techniques

(i) Preparative zone sedimentation centrifugation at neutral pH on preformed CsCl gradients was described by Burton & Sinsheimer (1965). In some experiments the technique was scaled up to permit the use of the Spinco SW 25.1 rotor.

(ii) CsCl equilibrium density gradient centrifugation of ØX DNA was described by Sinsheimer et al. (1962).

(iii) The sucrose gradient centrifugation analysis at pH 8.0 of ØX DNA was carried out as follows: Three 25 ml. 5 to 20% sucrose gradients (0.5 M NaCl, 5mM EDTA and 0.05 M Tris pH 8.0) were poured simultaneously at 2^{o}C. The gradients were allowed to stand at 2^{o}C for 1 to 2 hr. The samples, also at 2^{o}C, were then carefully layered onto the gradients, which were then loaded into the Spinco SW 25.1 rotor. The samples were centrifuged at 25,000 rev./min at 5^{o}C for about 13 hr in the Spinco Model L1 ultracentrifuge.

All gradients were collected dropwise from the bottom of the centrifuge tube into sterile shell vials.

(k) Radioactivity measurements

(i) Incorporation of ^{3}H-thymidine or ^{3}H-thymine into DNA in infected and uninfected cells was measured by removing a 1 ml. aliquot of the culture into a shell vial containing 0.1 ml. 5.5 N NaOH. The shell vial was then set at 37^{o}C for 17 to 20 hr. The sample was reneutralized by the addition of 0.1 ml. 6.0 N HCl. One ml. of cold 10% TCA was added to the sample at 0^{o}C. The sample was allowed to stand at 0^{o}C for 1 hr before collecting the precipitate by filtration through a Whatman glass filter disc. The filter was further rinsed with 30 ml. cold 2% TCA, dried and counted.

(ii) Labeled DNA was detected in gradients by addition of an aliquot of the sample to 0.5 ml. 0.05 M Tris pH 8.1, containing carrier calf

thymus DNA (100 μg); 1.0 ml. of cold 10% TCA was added and the sample was allowed to stand at 0°C for 1 hr. The precipitate was collected by filtration through a Whatman glass filter disc, rinsed with 10 ml. cold 2% TCA, dried and counted. The filter papers were placed into 10 ml. of a toluene-Liquifluor scintillator (New England Nuclear Corp.) and counted in a Beckman liquid scintillation counter.

3. RESULTS

(a) Phage maturation, lysis and cell death for infected cells at 15°C

When cells and phage are mixed under conditions proper for adsorption, but at 15°C, the phage attach to the cells but do not go into eclipse. This difficulty is circumvented by initiating the infection at 37°C, using starved cells in starvation buffer (Denhardt & Sinsheimer, 1965) as described in Materials and Methods. Under these conditions the phage attach to the cells and go into eclipse, but their DNA does not penetrate the cells; the nature of these early steps in the infection process will be discussed in a future publication (Newbold & Sinsheimer, manuscript in preparation). The eclipsed phage-cell complexes are then chilled and set at 15°C. The addition of one volume of nutrient medium precooled to the low temperature initiates the infection process at 15°C.

Figure 1 shows the time course of ØXam3 growth in infected cells at 15°C. It is evident that phage do not come out of eclipse under these

conditions and the titer of intracellular phage remains constant at about 0.1-0.3 PFU per cell (this value represents the background level of unadsorbed phage in this experiment) for beyond 24 hr. At 37°C phage start to come out of eclipse at about 10 min. However, the infected cells at 15°C retain the potential to mature phage since upon shifting an aliquot of such an infected culture to 30°C or 37°C, infectious intracellular phage rapidly appear in good yield. Such infected cells are unable to form colonies at 15°C. In a culture infected with a multiplicity of 5 ØX<u>wt</u> at 15°C (as previously described) about 0.5% of the cells retain the ability to form a colony at 15°C. Uninfected bacteria form colonies with equal efficiency at 15°C and 37°C. In liquid cultures uninfected bacteria grow exponentially at 15°C and show a generation time in KC broth of about 8 hr.

In order to determine whether the infecting viral DNA could penetrate the cell at 15°C, experiments were performed to monitor viral-induced lysis of the cells. The turbidity of cultures of <u>E. coli</u> C infected at high multiplicities at 15°C by <u>wt</u> and <u>am3</u> phage was measured. As shown in Figure 2, the turbidity of uninfected and <u>am3</u>-infected cells increased steadily at 15°C, whereas <u>wt</u>-infected cells showed the marked decrease characteristic of the lysis phenomenon. Since the lysis-defective mutant <u>am3</u> was unable to produce lysis at 15°C, that seen in <u>wt</u>-infected cultures can be considered to be due to the same process that induces lysis at normal temperatures.

The continued increase of turbidity in am3-infected E. coli C was described for the infection at 37°C by Hutchison & Sinsheimer (1966). The increase in turbidity did not reflect an increase in cell numbers, but rather in cell size. This same cell elongation is seen in the am3 infection at 15°C, and suggests that the viral-induced inhibition of cell division does not depend upon the ability of the infected cell to make infectious progeny phage. The lysis of wt-infected cells at 15°C further suggests that the ØX cistron I function was expressed and therefore that the viral DNA did penetrate into the host cell.

When a culture of E. coli C infected with a high multiplicity of ØXwt is sampled at the beginning of the infection at 15°C, and assayed at 15°C and 37°C for those residual cells that can form, after dilution and plating, a colony, the result is approximately identical for the two temperatures, viz. about 0.5% of the initial colony-forming cells survive as such. The same measurements performed on am3-infected E. coli C at 15°C reveal that at 37°C, again about 0.5% of the initial colony formers survive, but at 15°C the survival is about 8%. If in the normal infection each cell is killed by the combined action of lysis and inhibition of cell division, then these data could be interpreted to mean that the efficiency of lysis to induce cell death in a culture is independent of the temperature whereas the inhibition of cell division in am3-infected cells may be a less efficient cause of cell death at 15°C than at 37°C. Possibly an am3-infected

cell could with low probability escape from the inhibition of cell division at a late stage in the infection at 15°C and establish a colony at that temperature.

It is already apparent that infected cells shifted from 37°C to 15°C prior to the addition of nutrients do not make infectious phage after incubation with nutrients at 15°C. If the abortive infection were due to the malfunction of only an early event in the infection, then infected complexes shifted from 37°C to 15°C at late stages of the infection at 37°C might continue to mature phage at the low temperature. Such an experiment was performed with starved E. coli C cells infected with am3 to a multiplicity of 0.1 at 37°C in KC broth. During the infection at 37°C aliquots were removed and set at 0°C at times 0, 4, 8, 12, 16 and 20 min postinfection. The samples were then set to incubate at 15°C and the infection was allowed to continue. The six growth tubes were then sampled for measurements of intracellular phage during the incubation at 15°C. The data are given in Figure 3. They clearly indicate that phage maturation is rapidly and completely inhibited at 15°C even at the time when phage are being matured at the most rapid rate in the infection at 37°C. It is concluded that phage maturation cannot occur at 15°C and that the infection at 15°C might be abortive because of the malfunction of this late step in the infection process. However, the data so far presented do not preclude the possibility that the low temperature infection aborts at an earlier event in the infection.

(b) DNA synthesis in infected cells at 15°C

In the experiment presented in Figure 4, the incorporation of ^{3}H-thymidine into uninfected and am3-infected E. coli HF4704 cells at 15°C was measured. Prior to infection of the cells and during their starvation at 37°C, the culture was divided into two equal portions, and one portion was exposed to mitomycin C treatment. This treatment of hcr$^-$ cells permits a near normal ØX infection in the absence of host DNA synthesis (Lindqvist & Sinsheimer, 1967b). After mitomycin C treatment both portions of the culture were collected and resuspended in fresh starvation buffer, and then further subdivided into one subculture that remained uninfected and another that was infected with a multiplicity of 6.2 am3. The complexes were allowed 5 min at 37°C in starvation buffer to permit the phage to go into eclipse. They were then rapidly brought to 15°C and one volume of chilled nutrient medium containing both unlabeled and ^{3}H-thymidine was added. In all four cultures the incorporation was monitored as alkali-resistant, cold TCA-precipitable radioactivity.

In those cultures not exposed to mitomycin C, a marked inhibition of DNA synthesis was found as a result of ØX infection. Incorporation in those cultures given the mitomycin C treatment indicated that DNA synthesis was taking place at 15°C in the infected cells as a result of the infection. Since Lindqvist & Sinsheimer (1967b) showed that such incorporation in mitomycin C-treated hcr$^-$-infected cells reflected the synthesis of

ØX DNA, it can be concluded that some degree of ØX DNA synthesis is competent in the infection at 15°C. The incorporation of ^{3}H-thymidine into DNA in infected cells at 15°C reached a plateau in both mitomycin C-treated and untreated cultures by 15 hr of infection.

For the mitomycin C-treated, infected HF4704 cells the incorporation shows an initial lag for 1 hr and then proceeds at a linear rate corresponding to 5 RF molecules per cell per hr, as calculated from the specific activity of the labeled thymidine in the growth medium, until about 9 hr of infection; beyond this stage of the infection incorporation proceeds very slowly and by 15 hr some 50 equivalents of ØX RF are calculated to have been made. The increased level of DNA synthesis in infected cells not pretreated with mitomycin C over those given the treatment must reflect E. coli DNA synthesis and possibly also a greater yield of ØX DNA synthesis (in the absence of mitomycin treatment). If it is assumed that the difference between these two levels of incorporation represents only host DNA synthesis, then the amount of host DNA made after infection can be estimated from the specific activity of the label to be about 10% of the E. coli chromosome before net host DNA synthesis ceases.

In this same experiment, after 20 hr of am3 infection at 15°C in the presence of ^{3}H-thymidine, an aliquot of the culture that had been pretreated with mitomycin C was collected, the DNA extracted and analyzed

by preparative zone sedimentation centrifugation at neutral pH on a preformed CsCl gradient. The results of this analysis are given in Figure 5. The DNA labeled in the abortive infection shows the sedimentation profile characteristic of ØX RF; viral SS DNA appears not to be made at 15°C. The RF species I and II made in the abortive infection exhibit normal specific infectivity when assayed in the spheroplast system of Guthrie & Sinsheimer (1963).

The capability of am3 -infected cells to synthesize SS DNA upon a shift from 37°C to 15°C (after 20 min of the infection at 37°C) was examined next. As in the previous experiment, starved E. coli HF4704 cells were used, of which half were subjected to the mitomycin treatment; as before uninfected and am3-infected cultures (multiplicity was 6.0), pretreated and untreated with mitomycin C were monitored. After 20 min of infection in nutrient medium at 37°C the four cultures were chilled, ^{3}H-thymine was added to each, they were set at 15°C, and the incorporation was measured as alkali-resistant, cold TCA-precipitable radioactivity. The incorporation data are given in Figure 6. They indicate that ØX DNA synthesis is observed after such a temperature shift, at a linear rate for at least 5 hr. The greater incorporation in the infected mitomycin C-untreated culture, could again reflect host DNA synthesis or more likely a greater yield of ØX DNA synthesis; net host DNA synthesis would probably have been completely inhibited

by 20 min of infection at 37°C (Lindqvist & Sinsheimer, 1967a). There may be some ability for am3-infected cells to escape from this inhibition of host DNA synthesis at late times in the infection, when set at 15°C (consistent with the increased survival of am3-infected cells at 15°C); however, it is more likely that the mitomycin C treatment reduces the capacity of the host to sustain the full yield of ØX DNA synthesis in the infected cells. The rate of incorporation of ^{3}H-thymine into DNA measured in infected mitomycin C-treated HF4704 cells corresponds to approximately 10 RF molecules per cell per hr at 15°C as determined from the specific activity of the labeled thymine in the growth medium.

In this same experiment, after 2 hr of labeling the infected cells at 15°C, an aliquot of the culture that had been pretreated with mitomycin C was collected, the DNA extracted, and analyzed by preparative zone sedimentation centrifugation at neutral pH on a preformed CsCl gradient. The results of this analysis are given in Figure 7. The labeled DNA, made at 15°C after the temperature shift, has the sedimentation character of ØX RF; SS DNA is not made under such conditions.

These experiments suggest that in infected cells at 15°C the parental RF is made and that several rounds of RF replication can ensue until the RF pool reaches some saturation level. They further indicate that host DNA synthesis is inhibited in the abortive infection

and that synthesis of viral SS DNA is blocked at the low temperature. The transfer of infected cells from 37°C to 15°C, undertaken at late times in the infection at 37°C after net RF synthesis has ceased (Lindqvist & Sinsheimer, 1968) and SS DNA synthesis is under way, results in the immediate inhibition of SS DNA synthesis and the resumption of net RF synthesis; in these features the effect of the temperature shift-down resembles the effect of CAM in such an infection (Hutchison & Sinsheimer, 1966).

Estimates of the amount of DNA synthesized from the incorporation of ^{3}H-thymidine or ^{3}H-thymine at known specific activities in the growth medium are prone to error. For this reason a second approach has been taken to demonstrate conclusively that replication of the parental RF occurs in infected cells at 15°C. In this experiment starved E. coli HF4704 cells, pretreated with mitomycin C, were infected in starvation buffer at 37°C with a multiplicity of 1.1 ^{32}PD^{15}N ØXam3. Eight min were allowed for the phage to go into eclipse. The complexes were then chilled and set to 15°C and one volume of precooled nutrient medium was added that contained both unlabeled and ^{3}H-thymidine. The infection was allowed to proceed at 15°C for 10hr and then the cells were collected and the DNA extracted. The DNA was subjected to preparative zone sedimentation centrifugation at neutral pH on a preformed CsCl gradient. As in the experiment

described in Figures 4 and 5, no ^{3}H-labeled SS DNA was found; parental ^{32}P label from unpenetrated phage was found sedimenting as SS DNA . The ^{3}H-labeled DNA made de novo at 15°C gave the sedimentation pattern characteristic of RF; 60% of the ^{3}H cts/min as RF I and 40% as RF II . There were also ^{32}P cts/min sedimenting as RF I and RF II.

Those fractions of the gradient containing the ØX RF DNA were pooled and centrifuged to equilibrium in a preparative CsCl buoyant density gradient of mean density 1.72 g/ml. The results of this analysis are given in Figure 8. The ^{32}P-labeled parental RF molecules are seen to band at the same density as the ts$_{0}$h SS DNA infectivity marker, and are thus of hybrid density. Of the ^{3}H-labeled DNA made in the abortive infection at 15°C, a small fraction (about 7%) of the total ^{3}H cts/min is found at the position of the RF molecules of hybrid density, whereas the remaining 93% is found at the density expected of fully light RF. If it is assumed that the parental RF molecules of hybrid density are each composed of one ^{32}PD^{15}N-labeled "heavy" viral strand and one ^{3}H-labeled "light" complementary strand and that the fully light RF is ^{3}H-labeled in both strands, the ratio of ^{3}H cts/min in the fully light RF peak to that in the parental RF peak of hybrid density provides a measure of the amount of RF replication. In the normal infection at 37°C, only the parental RF molecules associated

with the cell membrane fraction engage in semiconservative replication of RF; progeny RF molecules free in the cytoplasm of the cell do not replicate (Knippers & Sinsheimer, 1968a). If the same is true in the replication of RF at 15°C, then the data in Figure 8 would suggest that each parental RF had, on the average, undergone 6.9 rounds of RF replication.

It is concluded that RF replication ensues in the abortive infection at 15°C. This also implies that the cistron VI gene product is functional in this infection (Levine & Sinsheimer, 1968).

A summary of the character of the ØX infection at 15°C is given in Table 1.

(c) Phage and DNA synthesis at low temperatures other than 15°C

Figure 9 shows the results of an experiment analogous to that described in Figure 5 but undertaken at the temperatures 15°C, 10°C and 5°C. However, in this experiment the am3 -infected (the multiplicity is 6.7), mitomycin C-pretreated HF4704 cells were incubated at the low temperature for 18 hr, and in the presence of ^{3}H-thymine The cells were then collected, the DNA extracted, and analyzed by preparative zone sedimentation centrifugation at neutral pH on preformed CsCl gradients. The sedimentation profiles indicate that RF can be labeled at 10°C and 5°C in decreasing amounts, and that the proportion of RF components I and II remains similar to that

found at 15°C.

It is known that ØX will form plaques when plated with sensitive cells at 25°C (Dowell, 1967). Therefore at some temperature between 15°C and 25°C SS DNA synthesis and phage maturation must become competent. Preliminary experiments were undertaken to determine what the minimum temperature is for SS DNA synthesis, and to ascertain if both processes have the same temperature threshold.

Starved E. coli HF4704 cells were infected at 37°C with am3 at a multiplicity of 14. The infected cells were allowed to develop at 37°C for 20 min -- a period sufficient for host DNA synthesis to be shut off -- and were then chilled to 0°C. ^{3}H-thymine was then added and the culture was divided into three equal portions. One subculture was set to incubate at 15°C, a second at 18°C, and the third at 22°C. The infected cells were collected after 1 hr of infection at the low temperature, the DNA extracted, and analyzed by preparative zone sedimentation centrifugation at neutral pH on preformed CsCl gradients. The results are shown in Figure 10. They indicate that the temperature threshold for SS DNA synthesis is close to 18°C.

Clearly SS DNA is made during the 1 hr pulse at 22°C. The total incorporation into ØX DNA in the pulse at 22°C is more than 3 times that for the pulse at 15°C, and 65% of it found as SS DNA. The pulse at 18°C reveals a total incorporation into ØX DNA only 10%

greater than for the pulse at 15°C, and the excess material is found sedimenting as a shoulder ahead of RF I. There is no distinct peak of SS material in the DNA pulse labeled at 18°C. Quite possibly intact SS DNA molecules may be represented in that material; however, the sedimentation profile is reminiscent of that found for the molecules described by Knippers, Razin, Davis & Sinsheimer (1969) and shown to be immediate precursors to SS DNA. It is of interest to note that the 1 hr pulse at 15°C seemed to preferentially label RF II in this experiment.

To determine at what temperatures progeny phage are made, phage were adsorbed to starved cells at 37°C in SVB and the complexes were shifted to a variety of lower temperatures for the subsequent development of the infection. Infections were analyzed at 15°C, 17°C, 20°C and 22°C, and the complexes were assayed for infectious, intracellular phage. The data are summarized in Table 2. The data indicate that complexes at 17°C can mature infectious progeny phage at a slow rate and with a poor yield.

The experiments described in this section suggest that RF DNA can be synthesized in infected cells at 5°C; also that the temperature thresholds for progeny phage maturation and SS DNA synthesis are both close to 17°C and could be identical.

(d) Can the RF molecules made in the abortive infection at 15°C serve as precursors to SS DNA after a shift to 37°C?

Previous publications have described the precursor role of progeny RF to SS DNA in infected cells at 37°C (Lindqvist & Sinsheimer, 1968; Knippers, Komano & Sinsheimer, 1968; Komano, Knippers & Sinsheimer, 1968; Dressler & Denhardt, 1968; Gilbert & Dressler, 1968; Knippers et al., 1969). The results described in a previous section demonstrated that semiconservative replication of the parental RF took place in the infection at 15°C, yielding a pool of progeny RF molecules of normal specific infectivity. To further probe the molecular precision of this replication and the potential of the RF molecules made at the low temperature, experiments were undertaken to examine the possible transfer of RF material made in the infection at 15°C into SS DNA after a shift of the infected cells to 37°C.

A culture of starved, mitomycin C-treated E. coli HF4704 was infected at 37°C in starvation buffer with am3 at a multiplicity of 4. After a period suitable for the eclipse of the phage, the infected cells were set at 15°C and an equal volume of nutrients was added to start the development of the infected cells at 15°C. The nutrients contained unlabeled thymine; no labeled thymine was yet present. The infected cells were incubated at 15°C for 1 hr and then ^{3}H-thymine was added. After a further 3 hr of infection at 15°C an aliquot of the culture was

removed, those cells collected and the DNA extracted. The remainder of the culture was set at 37°C and a large excess of unlabeled thymidine was simultaneously added as a chase. The culture was incubated at 37°C for 15 min, whereupon the cells were collected and the DNA extracted. The two DNA preparations were analyzed by sedimentation on sucrose gradients at neutral pH.

The results of this analysis are given in Figure 11 and are further summarized in Table 3. They indicate that approximately 40% of the RF material labeled during the period 1 hr to 4 hr at 15°C is transferred to SS DNA after the 15 min chase at 37°C and that the other 60% remains as RF. This implies that the temperature shift of 15°C to 37°C initiates the process of SS DNA formation found in the normal infection where viral strands are displaced from progeny RF II molecules and the remaining complementary strands persist and act as templates for the synthesis of more viral strands (Knippers et al., 1969). Knippers & Sinsheimer (1968b) have presented evidence that at least one other process can generate SS DNA from progeny RF; in this special case of SS DNA synthesis in cells infected by a coat protein mutant, viral SS DNA was matured into phage and the complementary strand was found to be degraded. This process does not occur in the experiment just described. The data in Table 3 indicate that the chase was not perfect; some 10% of further incorporation occurred during

the chase and this material is found as SS DNA.

The results of the experiment described in this section are taken to mean that viral SS DNA can be efficiently matured from essentially all of the members of the progeny RF pool made at 15°C, after the shift to 37°C. The complementary strands survive as such in RF without degradation and promote the further synthesis of viral DNA. That these RF molecules act so efficiently as precursors to SS DNA under the conditions of the temperature shift strongly suggests that the molecular detail of the RF replication process at 15°C is normal.

(e) Phage maturation and SS DNA synthesis in the presence of 100 µg/ml of CAM

The data presented so far indicate that in the abortive infection at 15°C a pool of progeny RF molecules accumulates that is of normal size (Knippers & Sinsheimer, 1968a) and which is fully competent in its precursor role to SS DNA after a suitable shift up in temperature. They further suggest that the products of ØX cistrons I (lysis) and VI (RF replication) can be translated and function. Reference to Table 1 shows also that a large quantity of SBP is present in the infected cells at 15°C; hence at least some coat protein is present. At this point it seemed not unlikely that in fact all gene products required for phage maturation might be present in adequate supply in the infection at 15°C

and that upon shifting to a temperature permissive for phage maturation further protein synthesis would be unnecessary. The following experiment was undertaken to test this possibility.

The abortive infection at 15°C was initiated as previously described using starved E. coli cells infected at a multiplicity of 3.5 am3. The infected cells were incubated in KC broth at 15°C for 10 hr and the culture was divided into two equal portions. CAM was added to one of those portions to a final concentration of 100 μg/ml. The cultures continued to incubate at 15°C for a further 10 min and were then set into an ice bath. From each of these cultures (CAM+ and CAM–) two subcultures were derived by dilution into KC broth at 37°C; one subculture contained 100 μg/ml. CAM, in the other CAM was absent. Samples were removed from the cultures throughout the infection to monitor the production of infectious intracellular phage. The results are shown in Figure 12. They indicate a rapid maturation of phage after the shift to 37°C which for the first 10 min is almost independent of the CAM treatment administered. Ten min after the temperature shift the cells contain on the average about 50 PFU per cell and for the complexes developing in the presence of 100 μg/ml. CAM this soon levels off at about 70 PFU per cell. Those cultures either never exposed to CAM or diluted away from it mature about 220 PFU per cell after 30-40 min at 37°C. The greater yield of phage in the cultures in which

CAM was absent is probably due to the continued synthesis of CAM-sensitive proteins vital for phage maturation, such as coat proteins.

The total amount of protein made in these infected cells in the initial 10 min period at 37°C (during which time a substantial yield of phage has been matured) was measured by administering a pulse of ^{3}H-L-leucine during that period in both CAM+ and CAM− cultures. The pulse was stopped by the addition of sodium azide and rapid cooling to 0°C. The cells were collected, washed and lysed by the lysozyme-EDTA method. Incorporation into total protein was measured by incubating an aliquot of lysate at 95°C with 7% TCA for 10 min; the samples were cooled, carrier calf thymus DNA was added and the precipitates were collected 1 hr later by filtration through Whatman glass filter discs. The ^{3}H-L-leucine incorporated into such TCA-precipitable material during the 10 min pulse was diminished 25-fold in the culture in which CAM was present at 100 μg/ml., i.e. the CAM reduced the rate of total protein synthesis to 4% of the control level.

It is concluded that the concomitant synthesis of CAM-sensitive proteins is not required for efficient phage maturation or SS DNA synthesis. It is further inferred that indeed the abortive infection at 15°C produces all of the materials required for phage synthesis and that the maturation process is blocked at the low temperature because one or more of these vital components is inactive.

An alternative interpretation for these results would be that at 15°C some vital product is not translated or transcribed, and its synthesis at 37°C is highly resistant to CAM. This possibility is considered most unlikely.

(f) Temperature shift-up experiments, in the presence of CAM, with ØX temperature-sensitive mutants

The proposed inactive vital product could be either a virus-coded protein whose role is obligatory for a successful infection or it might be a host-coded protein whose role is not essential to the host but vital for the infection. Such an inactive protein could be inactive for one of two reasons: (1) The protein might be unable to assume and maintain the proper conformation required for activity. (2) The nascent polypeptide may be perfectly able to adopt the proper conformation of the native protein but remain inactive at 15°C because of insufficient energy for its function. The first situation we denote "denatured and inactive," the latter, "native and inactive." Since abortively infected cells mature phage immediately when set to 37°C in the presence of CAM, the following two transitions must be entertained:

(i) 15°C, denatured and inactive to 37°C, native and active;

(ii) 15°C, native and inactive to 37°C, native and active.

Transition type (i) demands a protein folding event, whereas type (ii) does not.

Conditional lethal mutants of the temperature-sensitive type fall into the same two categories. The temperature-sensitive event may be the folding of the nascent polypeptide into the native conformation; alternatively, only the function of the native protein may be temperature sensitive. Temperature shift-up experiments in the presence of CAM following the regimen described in the previous section and using the ØXts mutants could be used to determine that ØX coded proteins of several cistrons are native in the abortive infection at 15°C.

For ØXts mutations, 30°C is the permissive temperature and 41°C is the restrictive temperature. E. coli C cells abortively infected with ØXts mutants are taken late in the infection at 15°C, after the addition of CAM to 100 µg/ml., and rapidly shifted to 30°C and 41°C by dilution into KC broth containing CAM. Intracellular phage measurements are made to determine if the complexes mature phage. All complexes should yield phage for the shift 15°C to 30°C. However, for the shift 15°C to 41°C two distinct types of infections can yield phage: (1) The protein coded by the mutant gene might be native and active at 15°C and its role in the infection completed at that temperature, i.e. its function might not be required after the temperature shifts in order that phage be produced. Or (2) if the virus coded protein must still function after the temperature shift, then only infections involving ts

mutants of the type that generate nascent proteins whose proper folding is temperature-sensitive, and which are already properly folded at 15°C, i.e. native, can yield phage. If the result of the 15°C to 41°C shift is positive, then it seems reasonable to conclude that the protein coded by that ØX cistron is native at 15°C. It is assumed that a protein which contains a temperature-sensitive mutation and which is in a denatured and inactive conformation at 15°C will upon shifting to 41°C in the presence of CAM remain nonfunctional at the high temperature. It is emphasized that this temperature shift analysis cannot indicate which cistrons are active or inactive at 15°C.

Such experiments have been performed using ØX*am3ts* double mutants and *E. coli* C, the nonpermissive host, to obviate problems caused by cell lysis. Such strains are available for ØX cistrons II, III, IV and VII. Cistrons I (lysis) and VI (RF replication) have already been shown to be native and active at 15°C. No mutants of the *ts* type are known as yet for the recently identified cistron VIII (F. Funk, personal communication).

A modification of the procedure just outlined was undertaken to test the cistron V protein at 15°C. The *am10* mutant in that cistron shows temperature-sensitive growth on the amber permissive host HF4714. To minimize the problem of lysis in the experiment, *am10*-infected HF4714 cells were taken after 5 hr infection at 15°C, and

after the addition of CAM, were shifted to 30°C and 41°C. Intracellular phage measurements were made after 10 min of infection at the high temperature, during which time cell lysis should not have been excessive. The results of these experiments are listed in Table 4.

The results indicate that all of the phage mutants were able to mature phage in the 15°C to 30°C temperature shift-up experiments in the presence of CAM. At least one ts mutant from each cistron tested is able to mature phage in such a 15°C to 41°C temperature shift experiment. The mutants am3ts4, am3tsγ, am3ts9, am10 and amts41D representing ØX cistrons II, III, IV, V and VII, respectively, all yielded unambiguously positive results. The double mutant am3ts79 also representing cistron III yielded an unambiguously negative result. The results for the experiment with am3 and am10 infections of E. coli HF4714 (amber permissive) are indicative of a positive result for the am10 temperature shift experiment; however, the poor yields of phage obtained per infected cell suggest that the problem of cell lysis is a limiting factor in this experiment. Attention is called to the fact that the maturation of am3 is itself temperature sensitive at 41°C; phage yields at 41°C are always less than those obtained at 30°C. Also the phage yields for the other mutants tested in this experiment indicate lower yields in infections at either 30°C or 41°C than for the corresponding situation for am3-infected cells.

It is concluded that the proteins coded by ØX cistrons II, III, IV, V and VII are in their native conformation at 15°C. Since the proteins coded by ØX cistrons II, III, IV and VII are structural components of the mature virus (Hutchison, 1969), after the temperature shift-up they must maintain their native conformation during the maturation of phage at 41°C, i.e. the mutations ts4, tsχ, ts9 and ts41D define proteins whose proper folding into the native conformation is temperature-sensitive and whose function, once the protein has folded, is not. The ts79 mutation of ØX cistron III, which yielded a negative result in the 15°C to 41°C temperature shift experiment, is considered to be of the type that gives rise to a protein whose function is temperature-sensitive.

4. DISCUSSION

(a) The character of the abortive infection

A detailed description of the character of the abortive infection by ØX174 at 15°C has been presented in the Results section of this paper. The results indicate that the infected cells are completely unable to synthesize SS DNA or mature phage at 15°C, and yet retain the potential to do so immediately upon an increase in temperature. All other viral-induced functions for which at present it is possible to assay, namely, RF replication, inhibition of host DNA synthesis, SBP and lysis, are found in the abortive infection at essentially normal levels.

The inhibition of host DNA synthesis found in the infection at 15°C (see Fig. 4) may be distinguished from that seen at 37°C. Lindqvist & Sinsheimer (1967a) reported finding an abrupt inhibition of host DNA synthesis after 12-14 min of infection at 40°C. At 15°C, no abrupt inhibition of host DNA synthesis is evident. The disparity in these observations might be a consequence of a diminished synchrony of the "shut off" event in the population of infected cells at 15°C or to a reduced efficiency of the process.

Temperature shift-down experiments in which infected cells are taken late in the normal infection at 37°C and shifted down to 15°C confirm the view that the cold-sensitive event(s) is (are) a late function(s). That the malfunction of some early event might also be in part responsible for the abortive infection seems unlikely in view of the rapidity and rate at which mature phage immediately appear in the temperature shift-up (15°C to 37°C) experiments -- 20 phage per cell per min. This rate of phage maturation is the same as that described by Hutchison & Sinsheimer (1966) for the normal am3 infection of the amber restrictive host. It might be expected that the cold-sensitive lesion would synchronize the abortively infected cells at 15°C, and that after raising the temperature the subsequent appearance of mature phage would occur at a more rapid rate and would endure for a shorter interval. This is not the case and would indicate that the

residual asynchrony in so-called synchronized ØX infections is intrinsic to the maturation steps in the infection .

The temperature shift-up experiments in the presence of 100 μg/ml. CAM -- in which the inhibitor was added to abortively infected cells after a prolonged incubation at 15°C, and then the culture transferred to 37°C -- proved to be most revealing. They demonstrated that SS DNA synthesis and phage maturation do not require the concomitant synthesis of CAM-sensitive proteins; at this level of the inhibitor, 100 μg/ml., it would seem likely that no concomitant synthesis of any protein is required at all, even though a pulse labeling experiment undertaken immediately after the shift to 37°C revealed a 4% residual level of total protein synthesis. These results contrast slightly with those reported by Hutchison & Sinsheimer (1966) which indicated that concomitant protein synthesis was required for phage maturation, but not for SS DNA synthesis. The results presented in this paper would indicate that in the infection at 15°C a pool of competent protein accumulates which is sufficient for complete phage maturation at almost the normal rate upon raising the temperature to 37°C, in the absence of further protein synthesis. No attempt has been made to detect the maturation of SS DNA into non-infectious subviral particles in these experiments.

Measurements of SBP had already indicated the presence of at

least one coat protein in infected cells at 15°C. The CAM temperature shift-up experiment, however, clearly implies that all of the ØX structural proteins must be present in good supply in the abortive infection. A more direct demonstration of this conclusion has recently been obtained by undertaking an acrylamide gel electrophoresis analysis of a lysate made from am3 abortively infected E. coli C cells at 15°C; the data (Newbold & Mayol, unpublished results) reveal the presence of the four ØX structural proteins (Edgell, Hutchison & Sinsheimer, 1969; Burgess & Denhardt, personal communication).

The CAM temperature shift-up experiments with the ØXts mutants further support the notion that the proteins coded by ØX cistrons II, III, IV, V and VII are present in the infection at 15°C and moreover adopt their native conformation. The data for the ts mutations in structural proteins of the native virus (ts4, ts$_\gamma$, ts79, ts9 and ts41D) also indicate that the majority of ts mutations impose their restriction only at the stage when the nascent polypeptide, containing an amino acid replacement defined by the mutation (Wittmann & Wittmann-Liebold, 1966), is folded into the native conformation. For the 5 ØX mutants tested, 4 were of this type; the exception was the ts79 mutation of cistron III which has been shown to give rise to both phage particles (Hutchison, Edgell & Sinsheimer, 1967) and to one spike

structural protein of altered electrophoretic mobility (Edgell, Hutchison & Sinsheimer, 1969). As mentioned in Results section (f) the failure of ts79 to mature phage in the CAM temperature shift-up experiment (15°C to 41°C) suggests that 41°C represents a denaturing condition for the mutant cistron III protein. It is known that ts79 phage stocks show the wild type sensitivity to heat inactivation (Sinsheimer, 1968). This would suggest that the mutant cistron III protein is in some way protected from denaturation once it has been incorporated into a spike on a phage particle.

In the CAM temperature shift-up experiment shown in Figure 12, the presence of the inhibitor does restrict the yield of infectious virus obtained after incubation at 37°C. The CAM^+ cultures yielded 70 PFU per cell; the CAM^- cultures yielded 220. This enhanced phage yield in CAM^- cultures is probably a consequence of the continued synthesis of CAM-sensitive proteins necessary for phage maturation, such as coat proteins. It is interesting to note that the specific infectivity of ØX stocks is usually about 20-30% (i.e. 20-30% of the virus particles are PFU). This would imply that perhaps some 200-350 virus particles were matured per cell in the CAM^+ culture at 37°C. The measurements of SBP given in Table 1, indicating 200 phage equivalents of SBP present per cell after 12 hr infection at 15°C, would suggest that perhaps the restriction on the amount of phage that can be

matured under these conditions is limited only by the amount of coat protein available.

The character of the abortive infection at 15°C could be interpreted in two ways:

(i) SS DNA synthesis is acutely cold-sensitive; without SS DNA molecules to mature, the terminal maturation process cannot operate.

(ii) The maturation of SS DNA molecules into phage is acutely cold-sensitive; the inability to mature phage in some way represses SS DNA synthesis.

Our present understanding of these steps does not at present permit us to favor one or other hypothesis. It is recognized that coat protein plays some role in SS DNA synthesis (Sinsheimer, 1968). Direct involvement of coat protein in SS DNA synthesis has been found recently (Knippers et al., 1969) in the binding of ØX specific viral antigens to DNA molecules that are intermediates in SS DNA synthesis.

It seems quite likely that SS DNA synthesis and maturation are parts of one integrated process -- a cold-sensitive maturation process.

(b) The nature of the abortive step in the infection at 15°C

These investigations have demonstrated the existence of a cold-sensitive function that limits the infection at 15°C. Three possibilities

are suggested as likely candidates for the cold-sensitive lesion:

(i) A virus-coded protein is "native" at 15°C, but its rate of activity at this temperature is too slow to sustain the infection.

(ii) Virus maturation may involve the formation of a DNA-protein complex and subsequent conformational changes which have an energy threshold infrequently reached at 15°C.

(iii) A host-coded protein present in uninfected cells, whose activity is essential to the infection at all temperatures but not to the host at 15°C, is inactive at 15°C.

Suggestion (i) is in accord with the observation that the character of the abortive infection at 15°C is identical to that produced by infections under nonpermissive conditions with conditional lethal mutants in ØX cistrons III, IV, V, VII and VIII. The protein specified by any one of these cistrons could be the cold-sensitive lesion. Suggestion (ii) has a precedent in the recently documented temperature-dependent conformational change critical to the in vitro assembly of the 30S ribosome (Traub & Nomura, 1969). It is quite likely that maturation of ØX174 involves protein-DNA and protein-protein binding reactions of a cooperative nature analogous to those occurring in the process of ribosome formation.

Suggestion (iii) is also possible, but it is difficult to envisage the function of such a host-coded protein. An hypothesis of this type could be based on the known temperature dependence of the properties of the E. coli DNA polymerase reaction in vitro. (The DNA polymerase activity required during the production of ØX SS DNA in vivo exhibits a 5' to 3' direction of polymerization (Knippers et al., 1969) identical to that of the host enzyme (Bessman, Lehman, Simms & Kornberg, 1958).)

When DNA polymerase is employed, utilizing ØX SS DNA as template in vitro (Mitra, Reichard, Inman, Bertsch & Kornberg, 1967; Goulian & Kornberg, 1967), the reaction at 15°C in the presence of an excess of deoxyribonucleoside triphosphates proceeds to a calculated level of one round of synthesis, i.e. as much product DNA is synthesized as was present as template DNA. If the temperature of the reaction mixture is now raised to 37°C, polymerization continues and the amount of product obtained increases several fold, limited by the amount of triphosphates available (Mitra et al., 1967; Newbold, unpublished results).

These data have been interpreted to mean that at the low temperature, DNA polymerase polymerizes nucleotides onto the terminal 3' -OH group on the primer strand only while there is free SS template DNA to be copied. This type of reaction has been called

"repair synthesis" (Richardson, Inman & Kornberg, 1964). The enzyme can only obtain template DNA to copy for further synthesis, as at 37°C, by displacing the DNA previously synthesized at the low temperature. This kind of DNA polymerase reaction is denoted "displacement synthesis." The two processes are schematically diagrammed in Figure 13. As elegantly shown by Knippers et al. (1969) ØX SS DNA synthesis in vivo demands, albeit well controlled, "displacement synthesis." The DNA polymerase of E. coli might undertake this role in vivo, and the infection may abort at that stage at 15°C because of the inability to sustain "displacement synthesis."

Of course, the semiconservative replication of E. coli DNA or ØX RF also requires a displacement reaction. If then the host DNA polymerase plays a role in semiconservative DNA replication, we must postulate that some other mechanism undertakes the displacement or denaturation of the template double-stranded DNA.

These postulates would make the temperature-dependent activity of the E. coli DNA polymerase vital for a successful infection by ØX, and, perhaps, nonessential to the host. Certainly viral gene products are involved in the production of SS DNA in vivo but the propensity for displacement synthesis is inherent in the host DNA polymerase-RF II DNA complex.

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FOOTNOTE

Abbreviations: SS, single-stranded; RF, replicative form, SBP, serum blocking power; CAM, chloramphenicol; TCA, trichloracetic acid; PFU, plaque forming units.

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Table 1

Character of the abortive infection at 15°C

Phenomenon associated with ØX infection	Function at 15°C
Attachment	+
Eclipse*	-
Penetration	+
Parental RF	+
RF replication	+
Inhibition of host DNA synthesis	+
SBP**	+
SS DNA synthesis	-
Phage maturation	-
Lysis	+

*
The block to the eclipse phenomenon at 15°C is circumvented by undertaking only the attachment and eclipse phases of the infection at 37°C and subsequently transferring cultures to 15°C for further analysis.

**
The SBP assays were performed by Robert Rohwer according to the procedure devised by Krane (1966). After 12 hr infection of E. coli C by am3 at 15°C, 200 phage equivalents of SBP were detected per infected cell.

Table 2

Growth of ØX174 at various low temperatures

Intracellular PFU found per infected cell during the infection at low temperature				
Temperature of infection	Time of removal of sample (hr)			
	0	3/4	6	17
15°C	0.28	0.22	0.31	0.26
17°C	0.30	0.27	0.64	3.6
20°C	0.25	0.28	1.05	5.4
22°C	0.22	0.18	11.0	38

Table 3

An analysis of the ØX DNA species labeled during a 3 hr period at 15°C and resolved by sucrose gradient centrifugation, before and after a 15 min chase at 37°C

	Before chase (^{3}H cpm)	After chase (^{3}H cpm)
SS DNA	-	27,620
RF I	32,620	17,990
RF II	19,110	11,720
Total	51,730	57,330

The data given in Table 3 are obtained from the sucrose gradient analyses given in Figure 11.

Table 4

The ability of cells abortively infected at 15°C with ØX ts mutants to mature phage after the addition of CAM (to 100 ug/ml.) and upon transfer to either 30°C or 41°C

Phage strain	Host	Time of infection at 15° C prior to the addition of CAM (hr)	PFU per cell immediately prior to the temperature shift-up	PFU per cell matured after 10 min at the high temperature 30°	41°
am3 (control)	C	12	0.4	75	48
am3ts4	C	12	0.3	54	22
am3ts	C	12	0.4	26	12
am3ts79	C	12	0 4	36	0.4
am3ts9	C	12	0.3	13	5.3
am3ts41D	C	12	0.3	35	21
am3 (control)	HF4714	5	0.3	3.8	2.6
am10	HF4714	5	0 1	2.5	1.1

FIGURE LEGENDS

Fig 1. The time course of phage replication in am3-infected E. coli C cells at 15°C.

E. coli C cells were grown in KC broth at 37°C to 1 x 10^8 cells/ml.; they were then washed once with SVB, concentrated 2-fold and incubated in SVB at 37°C for 90 min. ØXam3 was added to a multiplicity of 4.2; after a 5 min period at 37°C the culture was set into an ice bath and cooled to below 15°C. The culture was then set at 15°C and an equal volume of KC broth precooled to 15°C was added. 0.1 ml. aliquots of the culture were removed into 0.9 ml. 0.05 M tris pH 8.1 at 0°C for intracellular phage measurements as described by Hutchison & Sinsheimer (1966). After 12 hr and 28 hr of infection at 15°C, 0.1 ml. aliquots of the culture were removed and diluted into 10 ml. of KC broth at 37°C and further intracellular phage measurements made -●—●-, phage growth at 15°C; -▲—▲-, phage growth at 37°C.

Fig. 2. The lysis phenomenon in the abortive infection at 15°C.

E. coli C cells were grown in KC broth at 37°C to 1 x 10^8 cells/ml.; they were then washed once with SVB, concentrated 2-fold and incubated in SVB at 37°C for 70 min. The culture was divided into three equal portions. One portion was not infected, —+—+—;

the second was infected with a multiplicity of 10 ØXam3, —o —o—; the third was infected with a multiplicity of 9.5 ØXwt, —● —●—. The complexes were given 5 min at 37°C to permit the phage to go into eclipse, chilled to below 15°C, set at 15°C and an equal volume of KC broth at 15°C was added. The turbidity of the cultures was followed by measuring the absorbance at 600 mμ in a Bausch and Lomb Spectronic 20 colorimeter.

Fig. 3. The growth of ØXam3 in E. coli C at 15°C after a temperature shift-down from 37°C at various times during the infection at 37°C.

E. coli C cells were grown in KC broth at 37°C to 1 x 10^8 cells/ml., washed once with SVB, concentrated 4-fold and incubated in SVB at 37°C 90 min. The cells were then infected with am3 at a multiplicity of 0.1. 5 min later a 5 ml. aliquot was removed and set on ice, while to the remainder an equal volume of KC broth, prewarmed to 37°C, was added. At times 4 min, 8 min, 12 min, 16 min and 20 min post-infection, similar 5 ml. aliquots were removed from the culture at 37°C and set into an ice bath. These aliquots were subsequently set to incubate at 15°C and 0.4 ml. aliquots were removed from these cultures at various times, frozen and stored for later assay of intracellular phage as described by Hutchison & Sinsheimer (1966).

Sample Ⓐ , —o—o—, 0 min incubation at 37°C;
Ⓑ , —+—+—, 4 min; Ⓒ , —●—●—, 8 min;
Ⓓ , —⊙—⊙—, 12 min; Ⓔ , —⊡—⊡—, 16 min;
Ⓕ , —△—△—, 20 min.

Fig. 4. The incorporation of ^{3}H-thymidine into DNA in uninfected and am3-infected cells at 15°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymidine at 5 μg/ml. at 37°C to 1.6 x 10^{8} cells/ml. The cells were washed once in SVB, concentrated 4-fold and incubated in SVB at 37°C for 65 min. The culture was then split into 2 equal portions, and one portion was treated with mitomycin C as described by Lindqvist & Sinsheimer (1967b). The two subcultures now in fresh SVB, were each further split into 2 equal portions. One subculture of mitomycin C-treated cells, and one of untreated cells, remained uninfected in this experiment; the other two subcultures were each infected with ØXam3 to a multiplicity of 6.2. 5 min at 37°C was allowed for the phage to go into eclipse. The cultures were then chilled to below 15°C, set at 15°C, and an equal volume of TPA2x (plus glucose and unlabeled and ^{3}H-thymidine), precooled to 15°C, was added. Incorporation measurements were made as described in Materials and Methods. The growth medium for the cultures at 15°C contained 1.0 μC of ^{3}H-thymidine and

5.0 μg of total thymidine per ml. —□—□—, mitomycin C untreated, uninfected HF4704 cells; —⊙—⊙—, mitomycin C untreated, am3 - infected HF4704 cells; —●—●—, mitomycin C-treated, am3-infected HF4704 cells; —○—○—, mitomycin C-treated, uninfected HF4704 cells.

Fig. 5. Preparative zone sedimentation centrifugation of the DNA made in mitomycin C-treated, am3-infected E. coli HF4704 cells at 15°C.

The material was obtained from the experiment described in Fig. 4. 10 ml. of the am3-infected culture, pretreated with mitomycin C, were collected after 20 hr of infection at 15°C and the DNA extracted as described by Lindqvist & Sinsheimer (1967a). Half of the DNA recovered through this extraction procedure (0.2 ml.) was mixed with a small volume of ^{32}P ØXam3 SS DNA, and the mixture was layered onto a preformed 5 ml. CsCl gradient (Burton & Sinsheimer, 1965). The material was centrifuged at 37,000 rev./min at 20°C for 2.5 hr in the SW 50 rotor of the Spinco Model L2 ultracentrifuge. Two-drop fractions were collected from the bottom of the centrifuge tube into shell vials containing 0.4 ml. 0.05 M tris pH 8.1. 0.3 ml. aliquots of these fractions were precipitated and counted as described in Materials and Methods. —●—●—,

^{3}H cts/min; —o—o—, ^{32}P cts/min.

Fig 6. The incorporation of ^{3}H-thymine into DNA made in uninfected and am3-infected cells at 15°C subsequent to 20 min of incubation of the cultures at 37°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymine at 2.5 μg/ml. at 37°C to 2.0 x 10^{8} cells/ml. The cells were washed once in SVB, concentrated 4-fold and incubated at 37°C in SVB for 60 min. The culture was split into two portions and one portion was treated with mitomycin C. The two subcultures now in fresh SVB were each further subdivided into two subcultures. One subculture of mitomycin C-treated cells, and one of untreated cells remained uninfected in this experiment; the other two subcultures were each infected with ØXam3 to a multiplicity of 6.0. 7 min at 37°C was allowed for phage adsorption and then an equal volume of TPA2X (plus glucose and thymine), prewarmed to 37°C, was added to each culture. After 20 min infection at 37°C, the cultures were cooled to below 15°C, set at 15°C and ^{3}H-thymine was added. Incorporation measurements were made as described in Materials and Methods. The growth medium for the cultures at 15°C contained 10 μC of ^{3}H-thymine and 2.7 μg of total thymine per ml. —□—□—, mitomycin C untreated, uninfected HF4704 cells; —⊙—⊙—,

mitomycin C untreated, am3- infected HF4704 cells; —●—●—, mitomycin C-treated, am3-infected HF4704 cells; —o—o—, mitomycin C-treated, uninfected HF4704 cells.

Fig. 7. Preparative zone sedimentation centrifugation of the DNA made in mitomycin C-treated, am3-infected E. coli HF4704 cells at 15°C after a prior incubation at 37° for 20 min.

The material was obtained from the experiment described in Fig. 6. 25 ml. of the am3-infected culture, pretreated with mitomycin C were collected after 2 hr of incubation at 15°C and the DNA extracted. The DNA recovered through this extraction procedure was in a volume of 1.0 ml. 0.1 ml. of this DNA was mixed with a small volume of ^{32}P ØXam3 SS DNA, and the mixture was layered onto a preformed 5 ml. CsCl gradient. The material was centrifuged at 37,000 rev./min at 20°C for 2.5 hr in the SW 50 rotor of the Spinco Model L2 ultracentrifuge. Two-drop fractions were collected from the bottom of the centrifuge tube into shell vials containing 0.5 ml. 0.05 M tris pH 8.1. 0.4 ml. aliquots of these fractions were precipitated and counted as described in Materials and Methods. —●—●—, ^{3}H cts/min; —o—o—, ^{32}P cts/min.

Fig. 8. RF replication in the abortive infection at 15°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymidine at 2.5 μg/ml. at 37°C to 2.0 x 10^8 cells/ml. The cells were washed once in SVB, concentrated 6-fold and incubated at 37°C in SVB for 60 min before treatment with mitomycin C. The cells, in fresh SVB at 37°C, were infected with $^{32}PD^{15}N$ ØXam3 to a multiplicity of 1.1; 8 min was allowed for the phage to go into eclipse. The culture was then chilled to below 15°C, set at 15°C and an equal volume of TPA2X (plus glucose and unlabeled and ^3H-thymidine), precooled to 15°C, was added. The growth medium contained 10 μC of ^3H-thymidine and 2.5 μg of total thymidine per ml. After 10 hr of infection at 15°C the cells (120 ml.) were collected and the DNA extracted. The DNA was sedimented on a 25 ml. preformed CsCl gradient for 6 hr at 20°C in the SW 25.1 rotor of the Spinco Model L1 ultracentrifuge. 40 10-drop fractions were collected from the bottom of the centrifuge tube into empty sterile shell vials. 0.05 ml. aliquots of these gradient fractions were precipitated with TCA and assayed for radioactivity.

The fractions containing RF were combined (total volume was approximately 3.0 ml.) and CsCl was added to bring the solution to a mean density of 1.72 g/ml. A small aliquot of tsχh SS DNA infectivity marker was added to the solution. The material was centrifuged

in the SW 39 rotor at 15°C for 14.5 hr at 34,000 rev./min; the speed was then decreased to 30,000 rev./min and centrifugation was continued for a further 48 hr. 100 1-drop fractions were collected from the bottom of the centrifuge tube into shell vials containing 0.5 ml. 0.05 M tris pH 8.1. 0.02 ml. aliquots were removed from each gradient fraction to assay in the spheroplast system of Guthrie & Sinsheimer (1963) for the ts$_{\delta}$h infectivity marker. The remaining volume of these gradient fractions was precipitated and assayed for radioactivity as described in Materials and Methods. —●—●—, ^{3}H cts/min; —o—o—, ^{32}P cts/min.

The position in the buoyant density gradient of the peak of the ts$_{\delta}$h infectivity marker is indicated by an arrow. The RF material banding at the same position as the "light" SS DNA (L SS) is considered to be of hybrid density (Sinsheimer et al., 1962) and is denoted as HL (heavy-light) RF; the RF material banding higher in the gradient than the ts$_{\delta}$h L SS DNA is considered to be fully "light" RF and is denoted as LL RF.

Fig. 9. RF synthesis in infected cells at 15°C, 10°C and 5°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymine at 2.0 μg/ml. at 37°C to 1.8 x 10^{8} cells/ml. The cells were washed once with SVB, concentrated 4-fold and incubated at 37°C

in SVB for 80 min before administering the mitomycin C treatment. The cells, in fresh SVB at 37°C, were infected with am3 to a multiplicity of 6.7 8 min was allowed for the phage to go into eclipse at 37°C The culture was then chilled to 0°C in an ice bath, and an equal volume of TPA2X medium (plus glucose and unlabeled and ^{3}H-thymine), precooled to 0°C, was added. The growth medium contained 10 μC of ^{3}H-thymine and 1.1 μg of total thymine per ml. The culture was divided into 3 equal portions; one portion was set to incubate at 15°C, another at 10°C, and the third at 5°C. After 18 hr of infection, a 10 ml. aliquot of each culture was taken, the cells harvested and the DNA extracted. The DNA preparations, each in 0.2 ml., were mixed with a small volume of ^{32}P ØXam3 SS DNA, and the mixture was layered onto preformed 5 ml. CsCl gradients. The material was centrifuged at 37,000 rev./min at 20°C for 3 hr in the SW 39 rotor of the Spinco Model L1 ultracentrifuge. 2-drop fractions were collected from the bottom of the centrifuge tube into shell vials containing 0.5 ml. 0.05 M tris pH 8.1. The fractions were precipitated and counted as described in Materials and Methods.

—●—●—, ^{3}H cts/min; —o—o—, ^{32}P cts/min.

(a) 15°C; (b) 10°C; (c) 5°C.

The position in the gradients assigned to SS DNA and RF components I and II are indicated by arrows.

Fig. 10. SS DNA synthesis in infected cells at 15°C, 18°C and 22°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymine at 2.5 μg/ml. at 37°C to 2.1 x 10^8 cells/ml. The cells were washed once with SVB, concentrated 2-fold and incubated in SVB at 37°C for 60 min before am3 was added to a multiplicity of 14. 10 min was allowed for adsorption to occur at 37°C and then an equal volume of TPA2X (plus glucose and thymine), prewarmed to 37°C, was added. After 20 min of infection at 37°C, the culture was chilled to 0°C in an ice bath. Now ^{3}H-thymine was added to a final concentration of 10 μC per ml.; the total thymine concentration was 2.6 μg per ml. The culture was divided into 3 equal portions; one portion was set to incubate at 15°C, another at 18°C, and the third at 22°C. After 1 hr of incubation at these temperatures, a 10 ml. aliquot of each culture was taken, the cells collected and the DNA extracted. The DNA preparations, each in 0.2 ml., were mixed with a small volume of ^{32}P ØXam3 SS DNA, and layered onto preformed 5 ml. CsCl gradients. The material was centrifuged at 37,000 rev./min at 20°C for 2.33 hr in the SW 65 rotor of the Spinco Model L265 ultracentrifuge. 2-drop fractions were collected from the bottom of the centrifuge tube into shell vials containing 0.5 ml. 0.05 M tris pH 8.1. The fractions were precipitated and counted as described in Materials and Methods.

—●—●—, ^{3}H cts/min; —o—o—, ^{32}P cts/min.

(a) 15°C; (b) 18°C; (c) 22°C.

The positions in these gradients assigned to SS DNA and RF components I and II are indicated by arrows.

Fig. 11. The fate of the RF molecules made in the abortive infection at 15°C after transfer to 37°C.

E. coli HF4704 cells were grown in TPA medium (plus glucose) plus thymine at 20 μg/ml. at 37°C to 2.0×10^{8} cells/ml. The cells were washed once in SVB, concentrated 2-fold and incubated at 37°C in SVB for 70 min before treatment with mitomycin C. The cells, in fresh SVB at 37°C, were infected with am3 to a multiplicity of 4.0. 8 min was allowed for the phage to go into eclipse at 37°C. The culture was then chilled to below 15°C, set at 15°C, and an equal volume of TPA2X (plus glucose and thymine), precooled to 15°C, was added. The culture was incubated at 15°C for 1 hr with thymine at 1.0 μg/ml. After 1 hr of infection at 15°C ^{3}H-thymine was added to a final concentration of 25 μC per ml.; the total thymine concentration was now 1.2 μg/ml. The infection was continued at 15°C for a further 3 hr. 16 ml. of the culture was then chilled to 0°C in an ice bath. Another 20 ml. of the culture was transferred to 37°C and immediately 2.0 ml. of a thymidine solution (10 mg/ml. in TPA) was added. The culture was chased for

15 min at 37°C in the presence of excess unlabeled thymidine at a concentration of 0.9 mg/ml. After the 15 min chase at 37°C, 18 ml. of the culture was chilled to 0°C in an ice bath. The two aliquots at 0°C were centrifuged to collect the cells, and the DNA was isolated. The DNA preparations were each of volume 2.0 ml. at this stage. 0.5 ml. of each DNA sample was mixed with a small volume of ^{32}P ØXam3 SS DNA and further diluted with an equal volume of 0.05 M tris pH 8.1 before being layered onto a 25 ml. 5 to 20% sucrose gradient (0.5 M NaCl, 5 mM EDTA, 0.05 M tris pH 8.1). The samples were centrifuged at 25,000 rev./min at 5°C for 13.5 hr in the SW 25.1 rotor of the Spinco Model L1 ultracentrifuge. 30 10-drop fractions were collected from both gradients from the bottom of the centrifuge tube into empty sterile shell vials. The gradient fractions were precipitated and counted as described in Materials and Methods.

—●—●—, ^{3}H cts/min; —o—o—, ^{32}P cts/min.

(a) Sample labeled with ^{3}H thymine for the period of 1 to 4 hr postinfection at 15°C.

(b) Same as for (a) but subsequently chased at 37°C with excess thymidine for 15 min.

The positions in these gradients assigned to SS DNA and RF components I and II are indicated by arrows.

Fig. 12. Phage maturation in the presence of 100 μg/ml. CAM.

E. coli C cells were grown in KC broth at 37°C to 2.0×10^8 cells/ml. The cells were collected by centrifugation, washed once with SVB, concentrated 2-fold and incubated in SVB at 37°C for 75 min. The culture was then infected with ØXam3 to a multiplicity of 3.5. 8 min were permitted for the phage to go into eclipse and the culture was then chilled to below 15°C, set at 15°C, and an equal volume of KC broth, precooled to 15°C, was added. After 10 hr of infection at 15°C the culture was divided into 2 equal portions. To one portion of the culture, CAM was added (dissolved in KC broth). The cultures continued to incubate at 15°C for 10 min and were then set into an ice bath. From each of these cultures (CAM^+ and CAM^-), two subcultures were derived; one by 10^4-fold dilution into KC broth at 37°C, the other by identical dilution into KC broth at 37°C, containing CAM at 100 μg/ml.

The two cultures at 0°C in the ice bath were returned to incubate at 15°C. Intracellular phage measurements were made on the two cultures at 15°C, and the four subcultures at 37°.

—O—O—, CAM^+ at 15°C; —+—+—, CAM^- at 15°C;

—⊙—⊙—, CAM^+ at 15°C and diluted into CAM^+ at 37°C;

—△—△—, CAM^+ at 15°C and diluted into CAM^- at 37°C;

—⊡—⊡—, CAM^- at 15°C and diluted into CAM^+ at 37°C;

—●—●—, CAM^- at 15°C and diluted into CAM^- at 37°C.

Fig 13. Schematic representation of the in vitro E. coli DNA polymerase reaction utilizing ØX viral DNA as template and a small oligonucleotide as primer.

"Repair" synthesis converts the circular SS DNA template into a synthetic RF II molecule. Further "displacement" synthesis yields a more complex product.

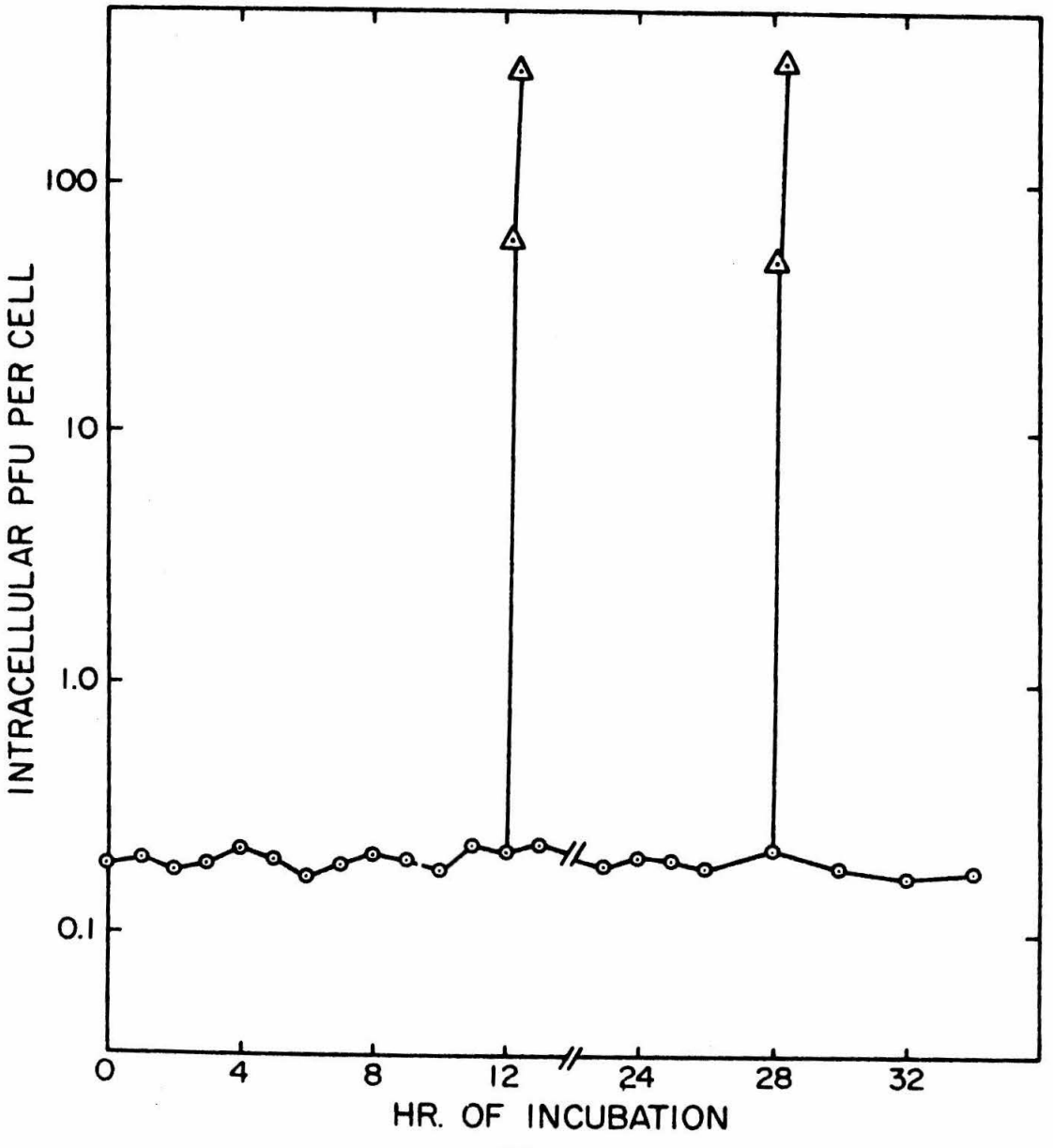
INTRACELLULAR PFU PER CELL
100
10
1.0
0.1
0
4
8
12
24
28
32
HR. OF INCUBATION

Fig. 1

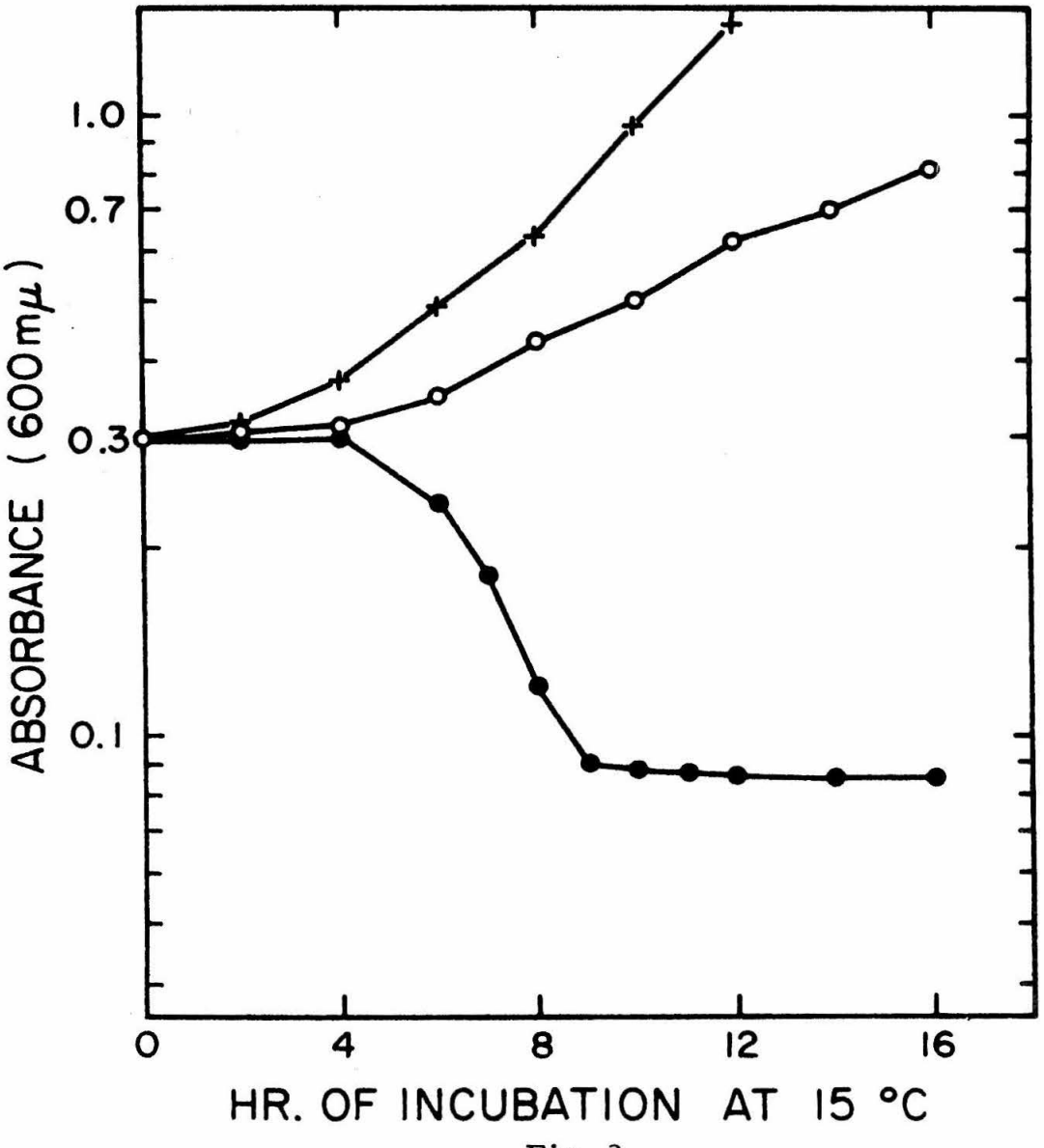

Fig. 2

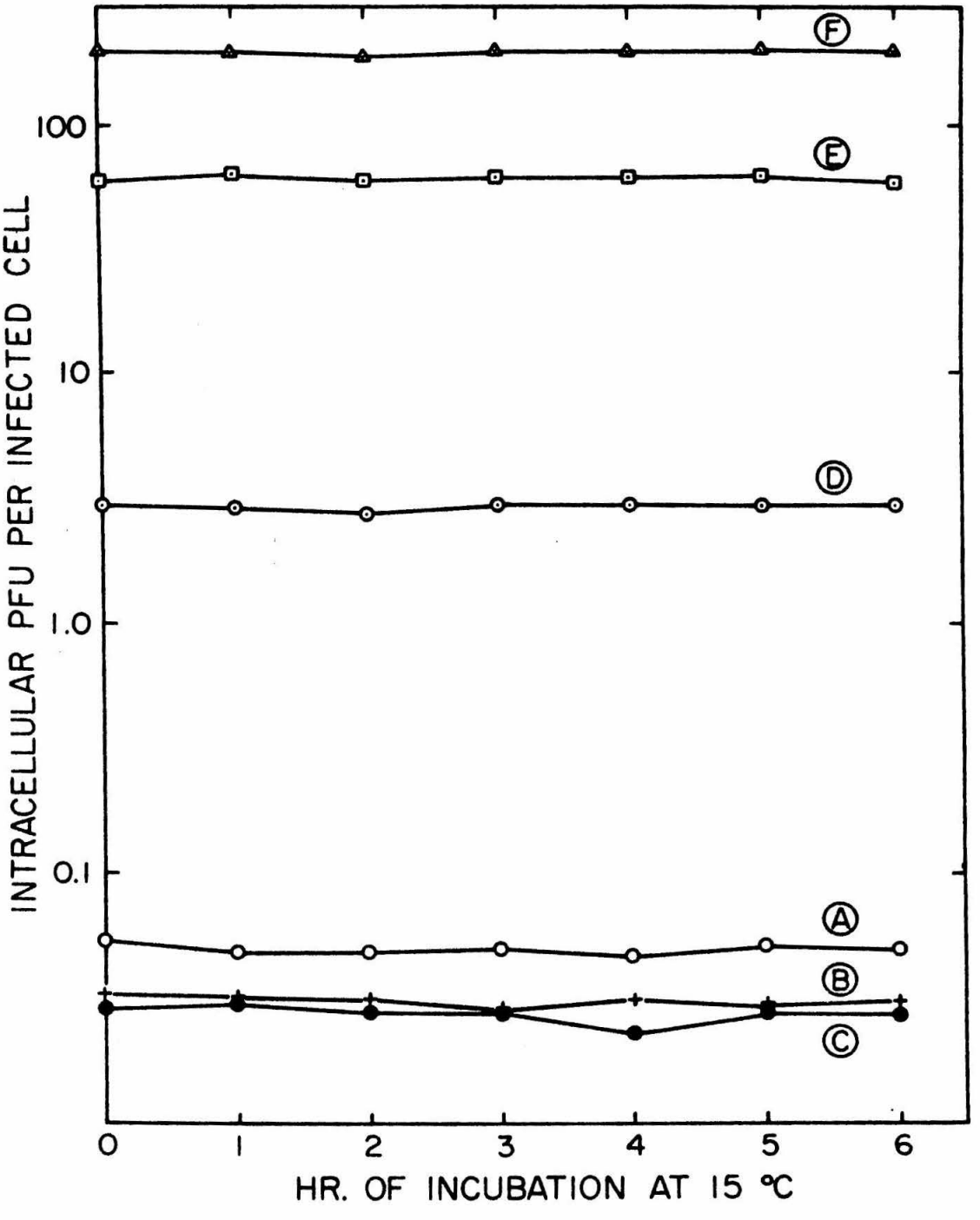
INTRACELLULAR PFU PER INFECTED CELL
100
10
1.0
0.1
F
E
D
A
B
C
0
1
2
3
4
5
6
HR. OF INCUBATION AT 15 °C

Fig. 3

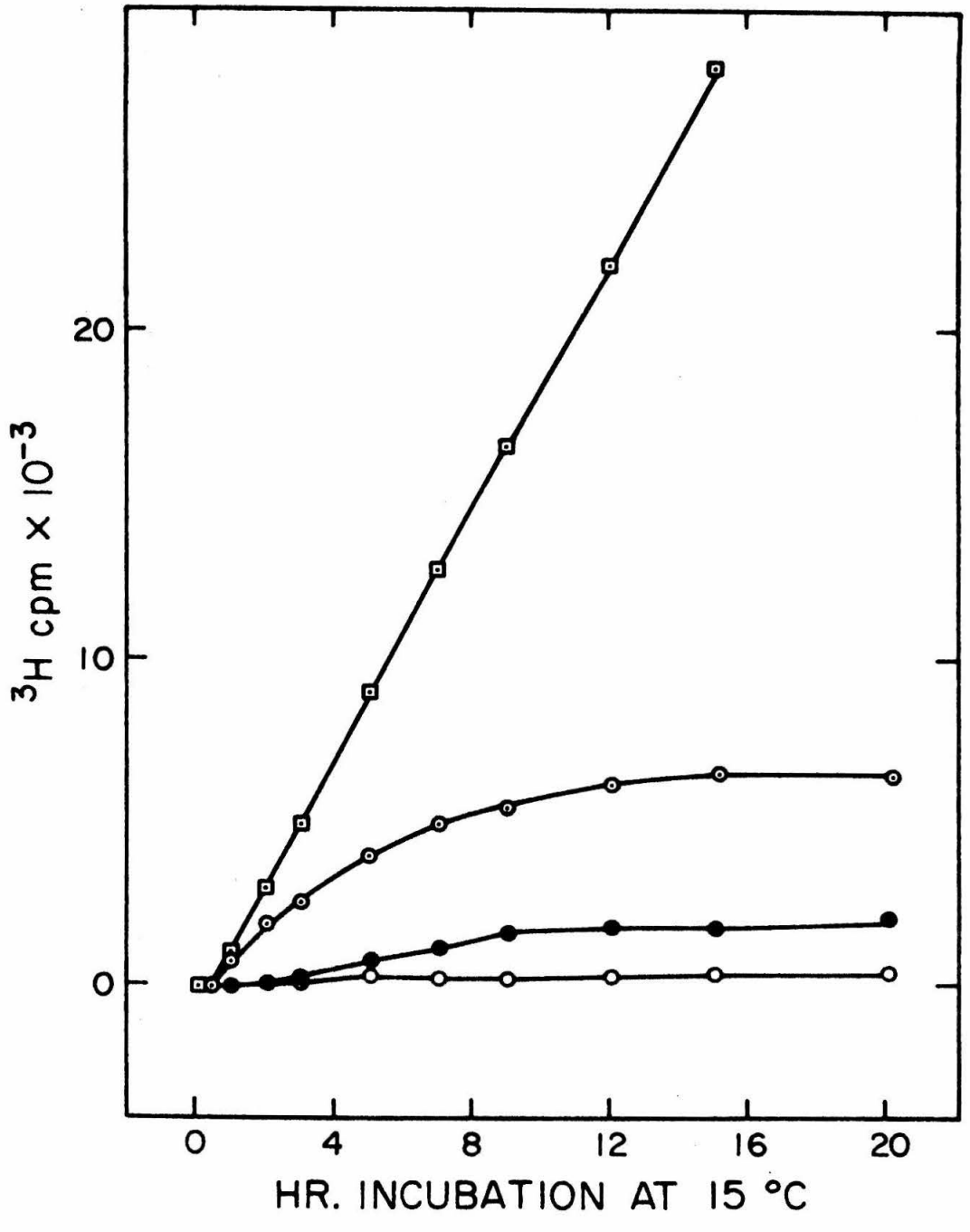

Fig. 4

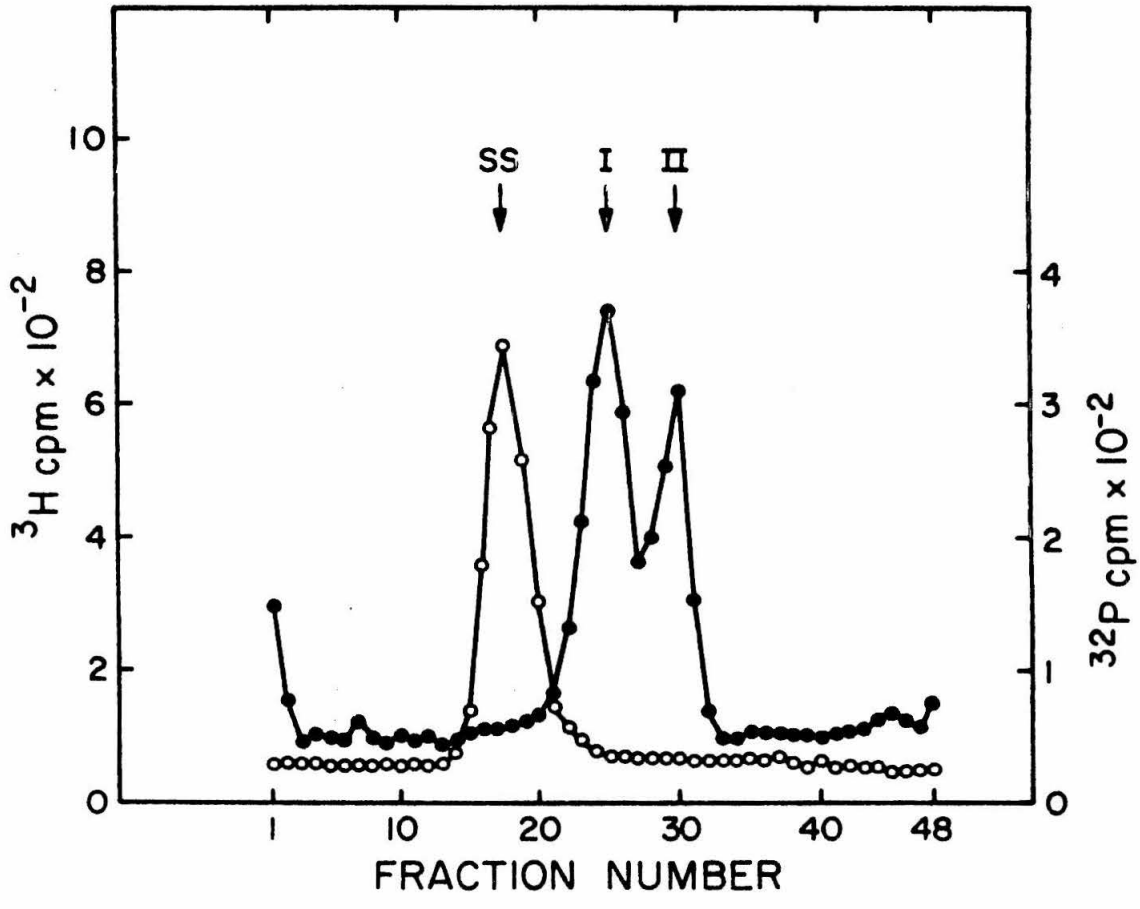
SS
I
II
^{3}H cpm x 10^{-2}
^{32}P cpm x 10^{-2}
0
2
4
6
8
10
0
1
2
3
4
1
10
20
30
40
48
FRACTION NUMBER

Fig. 5

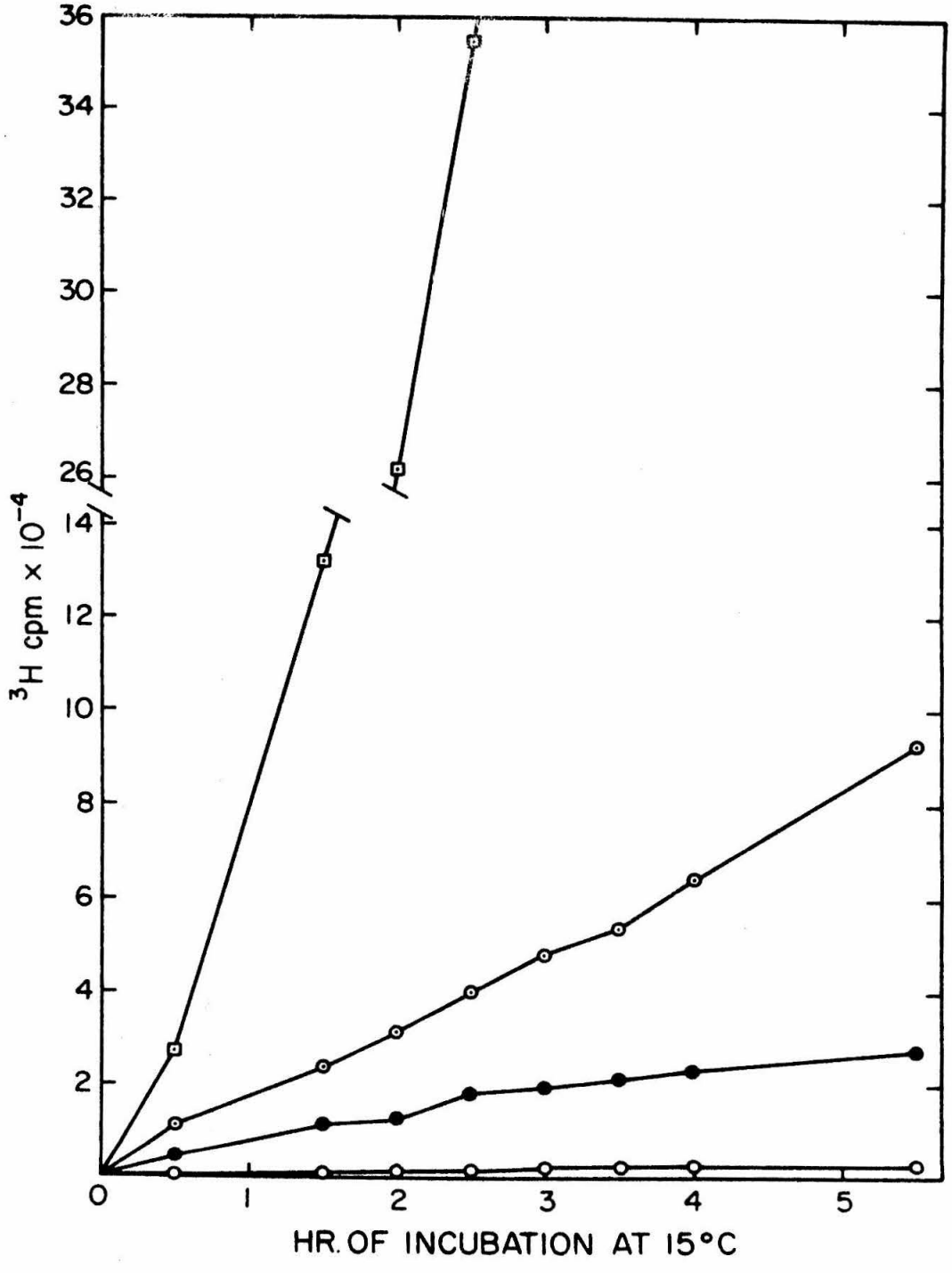
36
34
32
30
28
26
14
12
10
8
6
4
2
0
1
2
3
4
5
^{3}H cpm x 10^{-4}
HR. OF INCUBATION AT 15°C

Fig. 6

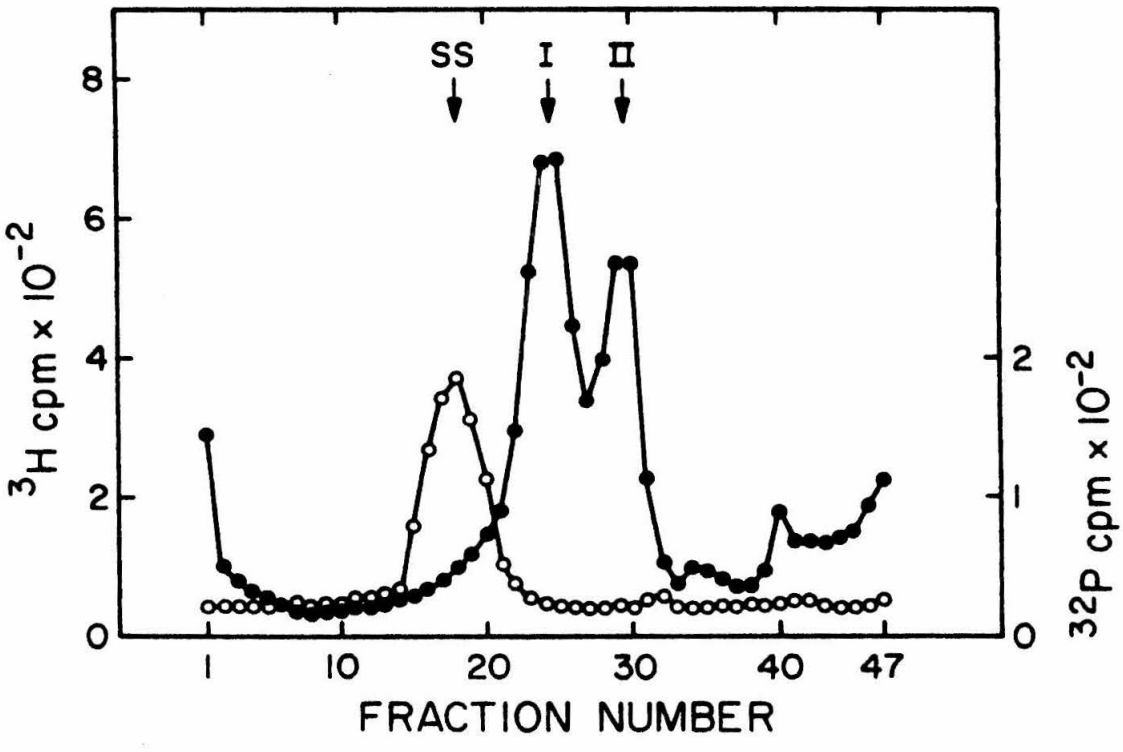
SS
I
II
8
6
4
2
0
^{3}H cpm x 10^{-2}
2
1
0
^{32}P cpm x 10^{-2}
1
10
20
30
40
47
FRACTION NUMBER

Fig. 7

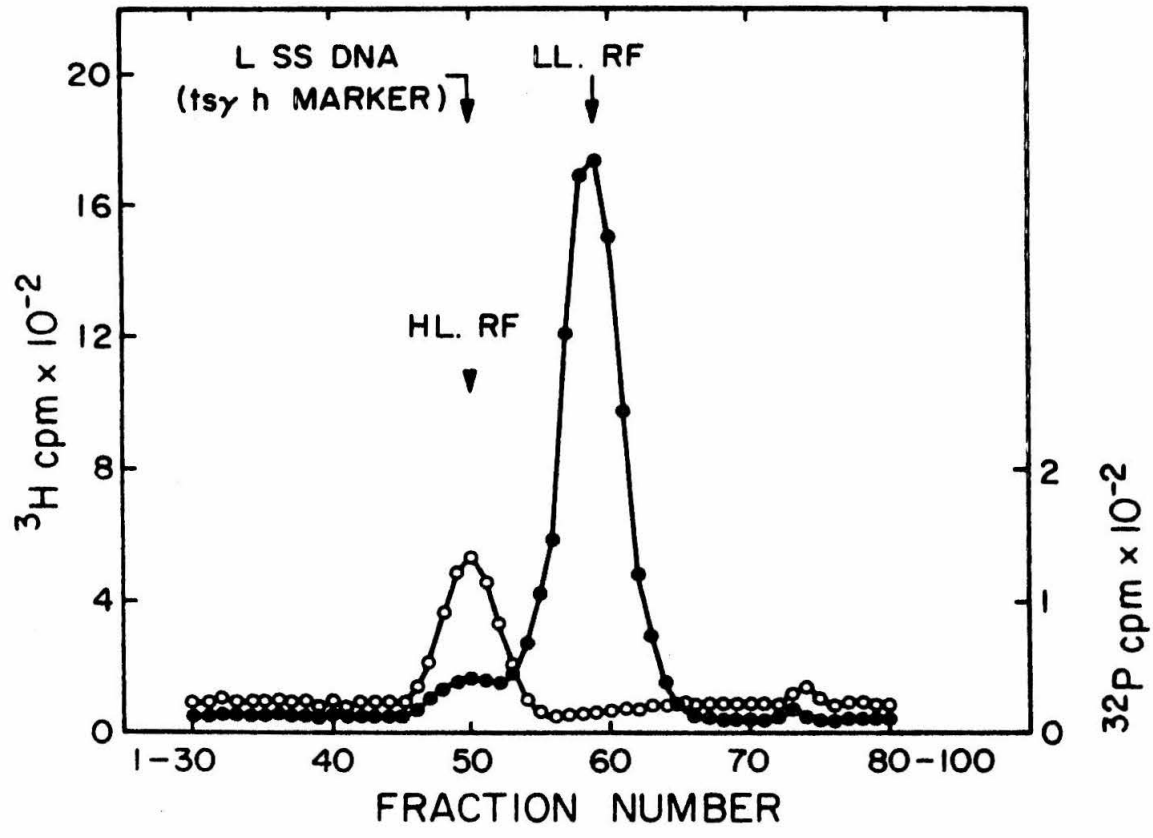
L SS DNA
(tsy h MARKER)
LL. RF
HL. RF
20
16
12
8
4
0
^{3}H cpm x 10^{-2}
2
1
0
^{32}P cpm x 10^{-2}
1-30
40
50
60
70
80-100
FRACTION NUMBER

Fig. 8

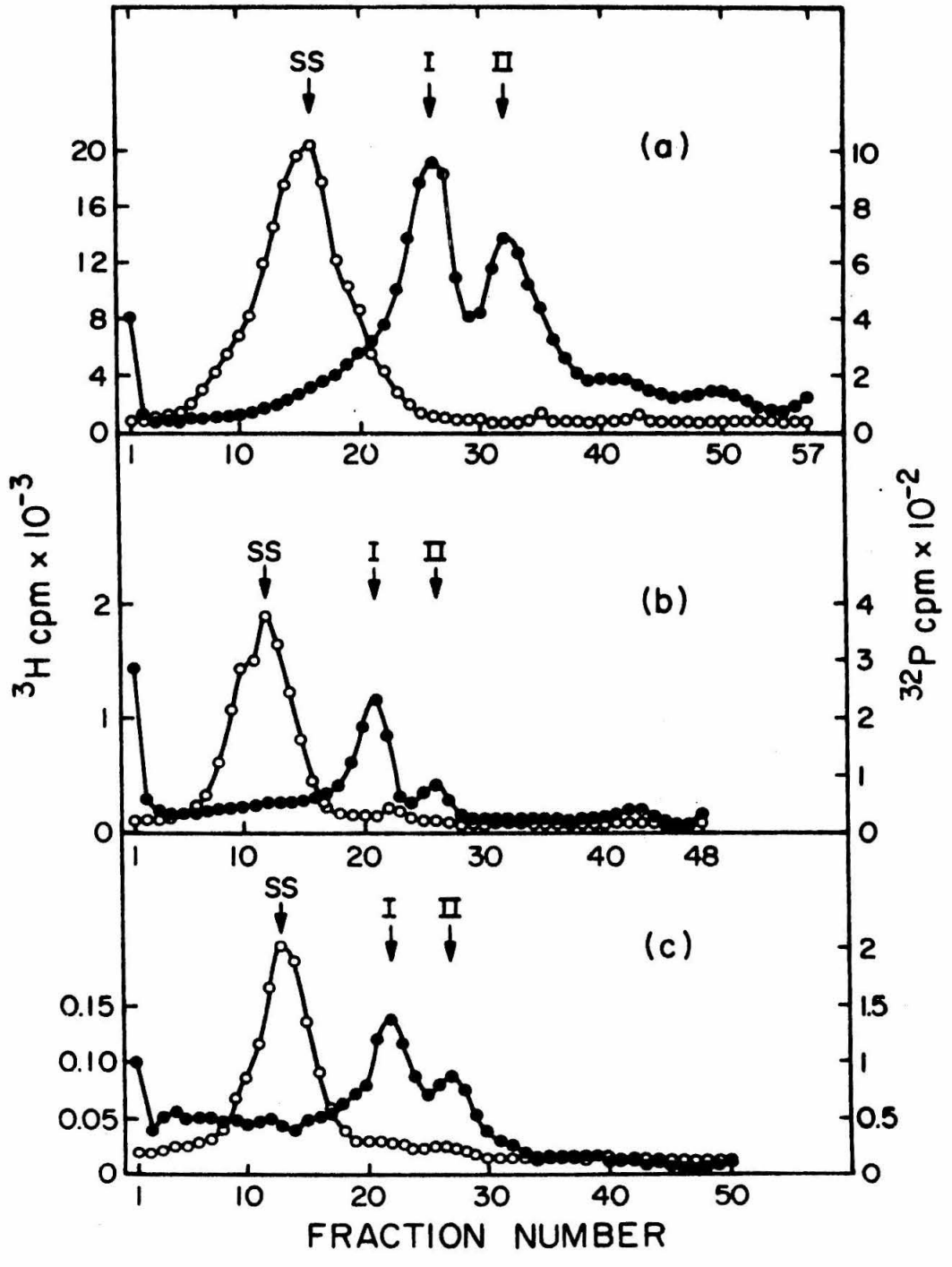

Fig. 9

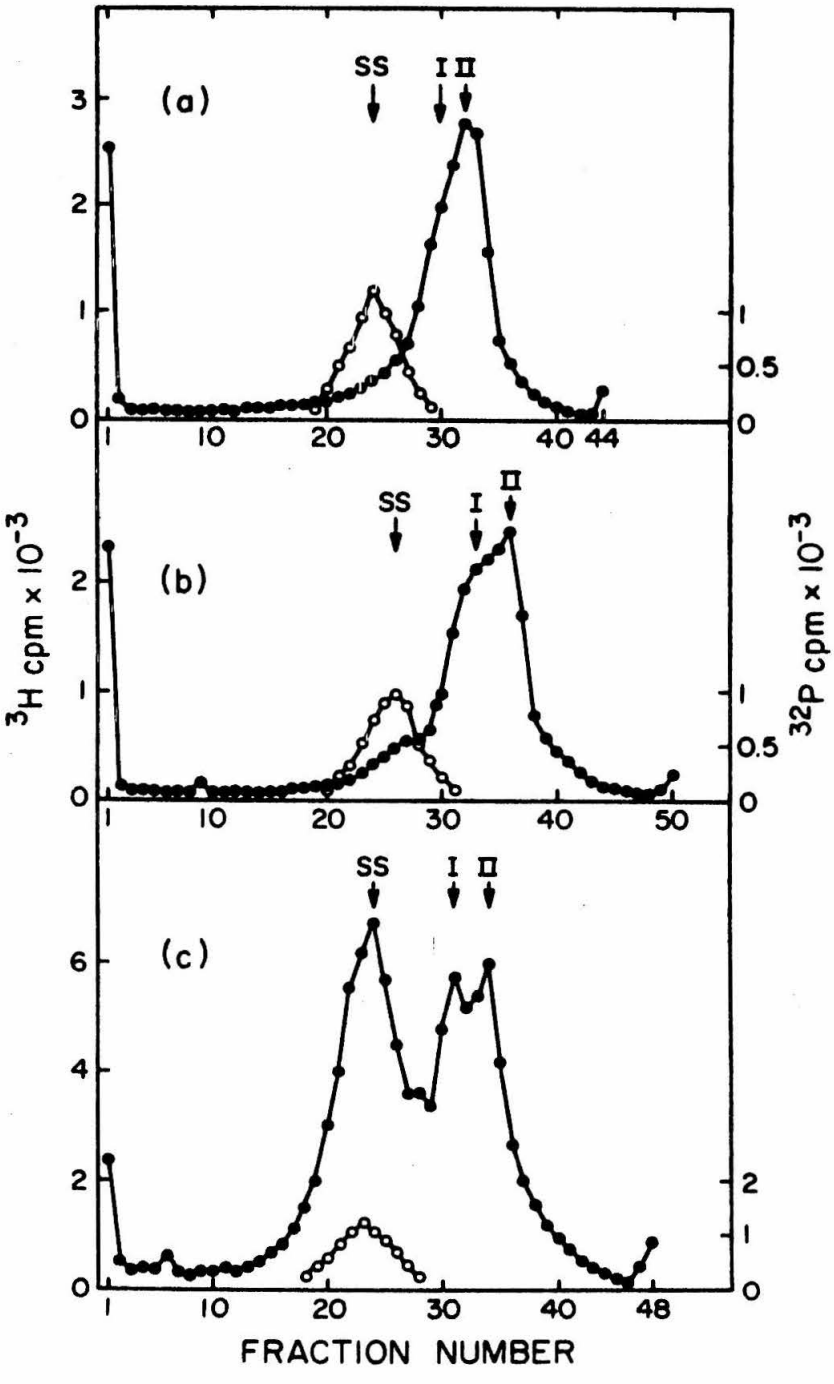

Fig. 10

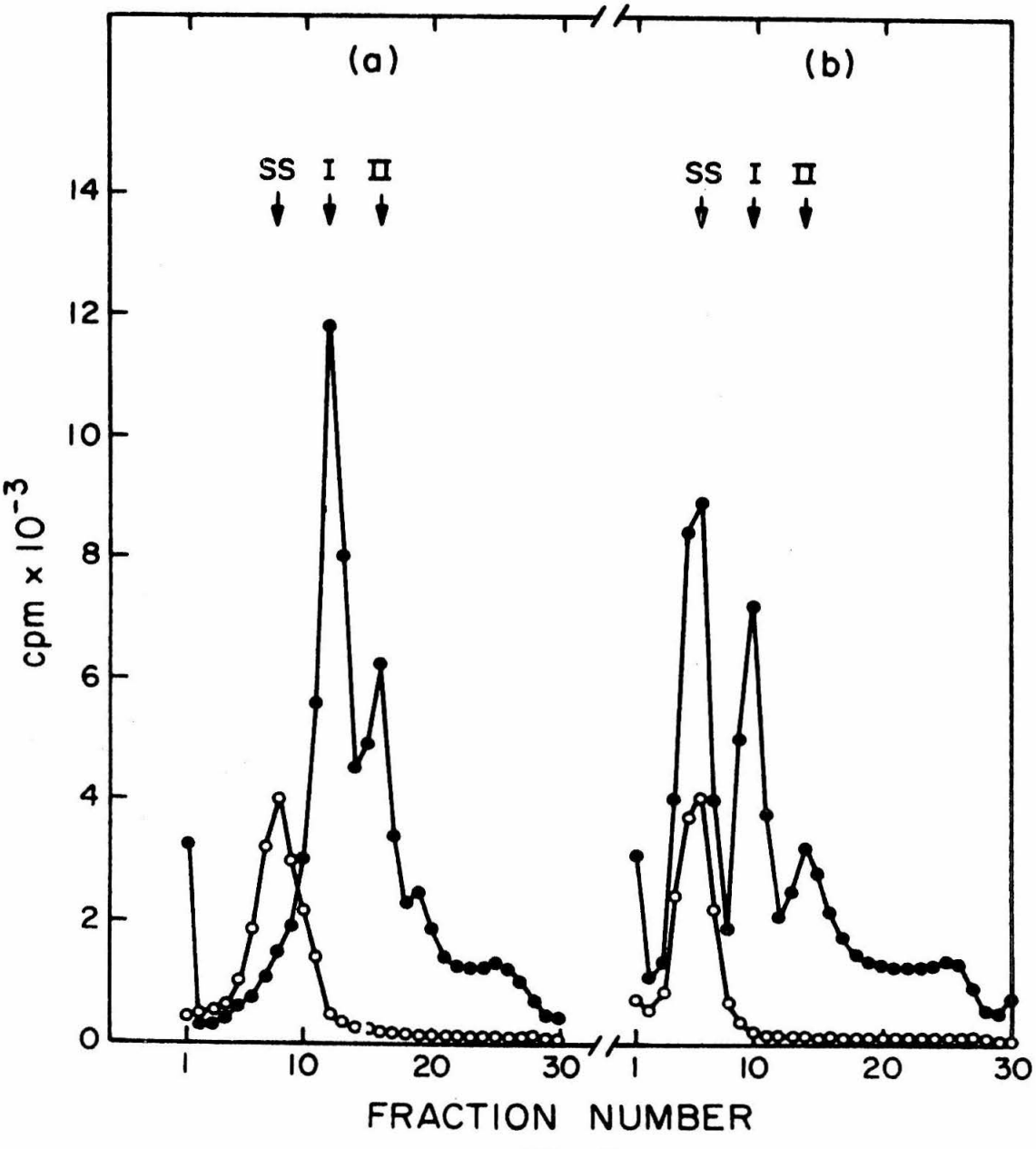
(a)
(b)
SS I II
SS I II
14
12
10
8
6
4
2
0
cpm x 10⁻³
1
10
20
30
1
10
20
30
FRACTION NUMBER

Fig. 11

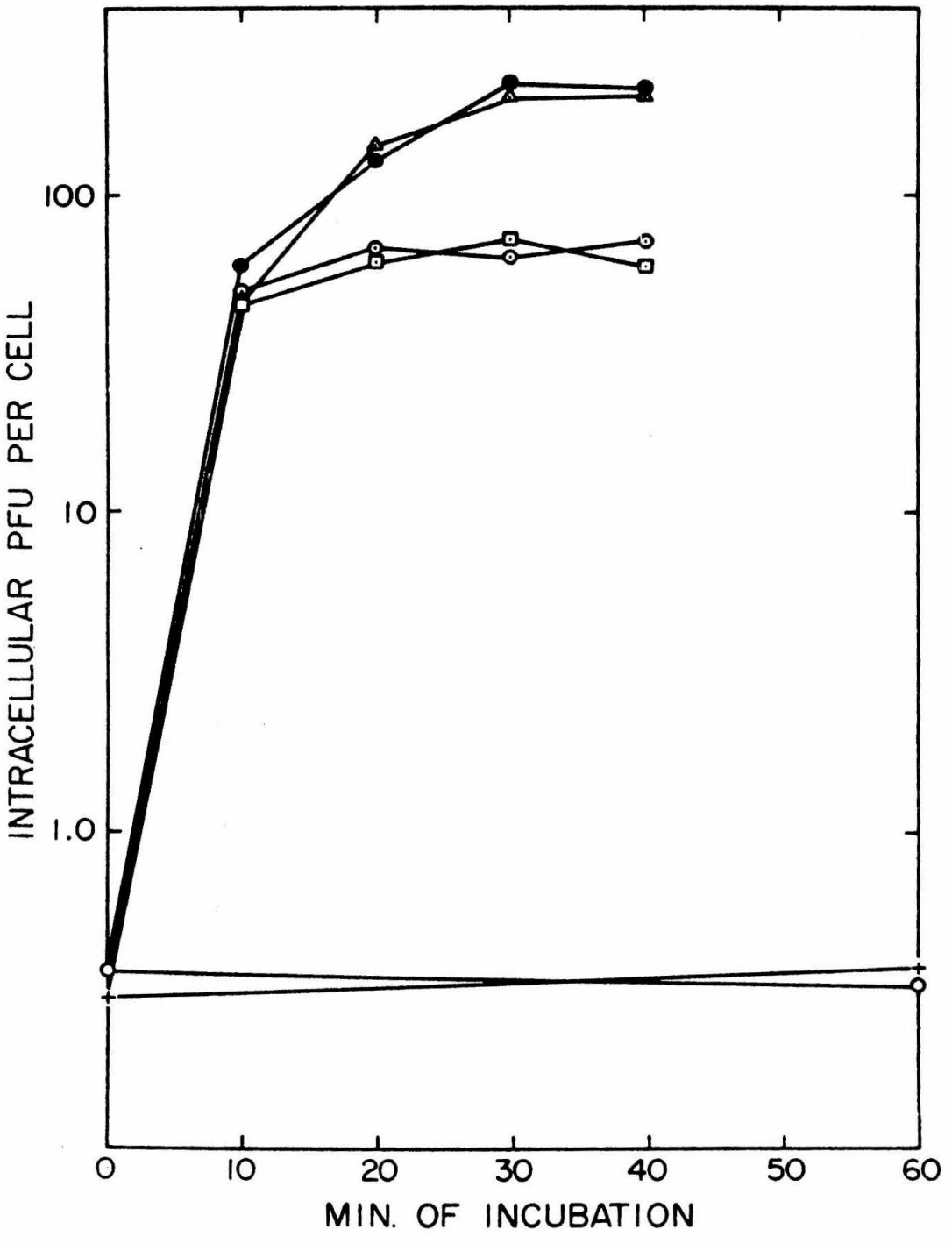
INTRACELLULAR PFU PER CELL
100
10
1.0
0
10
20
30
40
50
60
MIN. OF INCUBATION

Fig. 12

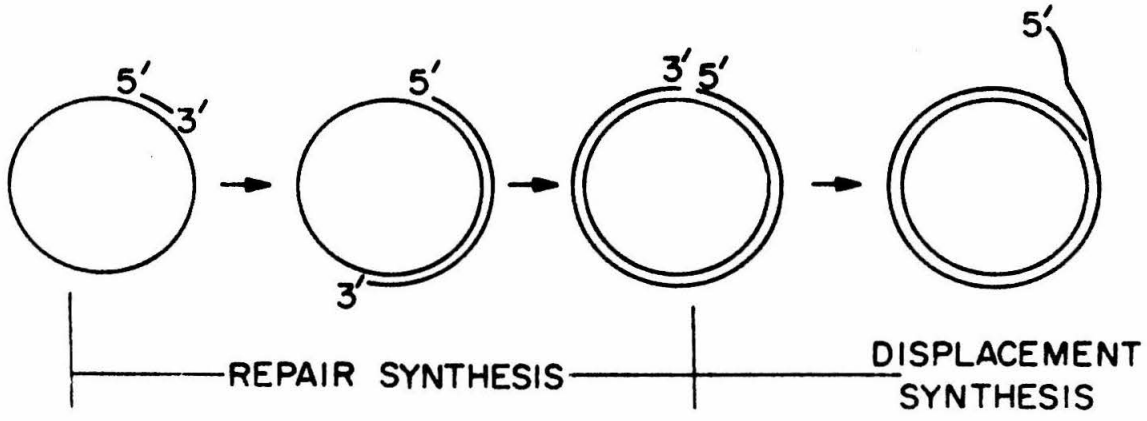

Fig. 13

I.3 Discussion

For the experiments reported in the previous section of this thesis it is difficult to evaluate the effect, if any, of the physiological damage experienced by the host due to its exposure to low temperatures for two reasons. (1) The cold-sensitive lesion that restricts the growth of E. coli at low temperature remains unidentified. (2) It is not clear that the temperature shift-down regimen used in the investigation of ØX infection at low temperatures would necessarily cause any physiological damage to the cells The range of 'normal' temperature extends down to 15°C for cells grown in complex media, but only to 20°C for glucose-minimal media (Ng, 1963). It seems probable that the experiments concerning DNA synthesis in ØX-infected cells at low temperatures which were performed in the TPA medium (glucose-pyruvate-minimal medium, plus amino acids) fall into the 'low' temperature range. However, most of those experiments have also been carried out in KC broth at 15°C with essentially the same results (Newbold, unpublished experiments).

The accomplishment of the temperature shift-down to 15°C (or below) in most experiments involved first the chilling of the culture to below 15°C, sometimes even to 0°C and then permitting the culture to warm to the desired temperature. Again it is not apparent from the data in the literature that this protocol would harm the cells and

therefore it cannot be excluded that ØX infection aborts at 15°C as a result of an altered physiology of the host at low temperature.

After their study of the cold-sensitive mutant cell K-II-27, O'Donovan and Ingraham (1965) observed that the conformational changes of allosteric proteins might be a likely site for cold-sensitive lesions. As proposed in the previous section (I.2) protein-nucleic acid and protein-protein interactions of a cooperative nature might more generally be the basis of cold sensitivity. However, all such interactions need not be cold-sensitive. It would seem quite probable that the assembly of whole tails to intact heads of phage λ involves cooperative interactions and yet the reaction in vitro is reported to be almost as rapid at 0°C as at 37°C (Weigle, 1966).

Attempts at obtaining ØX174 synthesis by in vitro complementation experiments modeled after those of Edgar and Wood (1966) have been unsuccessful (Hutchison, unpublished experiments). The investigations in section I.2 have documented how infected cells at 15°C contain all the precursors to mature phage viz. competent coat proteins and RF DNA. The system naturally suggests itself as a means to obtain and study in vitro ØX SS DNA synthesis and phage maturation. Preliminary attempts to synthesize infectious phage from lysates of infected cells grown at 15°C have been unsuccessful (Newbold, unpublished experiments); however, the system merits further study.

The results in section I.2 indicate that the temperature thresholds for SS DNA synthesis and phage maturation are close to 17°C. The results to be presented in section II. 3 indicate that the temperature threshold for the process of phage eclipse is also 17° to 18° C. It is conceivable that one particular cold-sensitive protein-DNA or protein-protein interaction restricts all three phenomena at low temperature.

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PART II

THE EARLY STAGES IN THE PROCESS OF INFECTION BY BACTERIOPHAGE ØX174

II.1 Introduction

Although subviral particles or defective virions may affect the host cell, the basic concern of virology is what happens when viral nucleic acid interacts with a cell; this interaction constitutes "infection". The attainment of this condition usually requires several processes to occur before the viral nucleic acid is exposed to that part of the cell in which it can exert its specific effect. These processes comprise the invasion mechanism for the virus-host system and begin with the specific attachment of the virion to the exterior portions of the cell. The dissolution of the integrity of the virion during the invasion of the host results in the eclipse of viral infectivity so characteristic for all viruses.

The basic strategy of the invasion process at the cellular level is to attach stably to the host, and then to penetrate. Many variations to this theme exist; nonetheless, it is possible to distinguish two types of invasion, viz (i) penetration by "injection" of viral nucleic acid or, (ii) by "engulfment" of the virion. The former class of invasion mechanism involves the release of the viral nucleic acid (perhaps accompanied by other minor components of the virion) into the cell at a peripheral cellular site; the viral capsid remains outside of the cell. This type of invasion mechanism is certainly the dominant, and perhaps the exclusive mode for invasion by the bacterial viruses.

Penetration by engulfment is the invasion mechanism characteristic of the animal viruses. In this process the virion is usually taken into the cell in a vacuole and there undergoes partial or complete uncoating leading to exposure of the viral nucleic acid within the cell. The invasion mechanisms of insect and plant viruses (at the cellular level) remain completely obscure and provide, as yet, essentially no information on these phenomena.

The invasion process for the bacteriophage systems exhibits a diversity commensurate with their wide range of structural complexity. The phages that contain a relatively larger quantity of nucleic acid, bear tails, which seem to be organelles for both the specific attachment of the phage to, and the transfer of viral DNA into, the host bacterium. Phages of relatively small genetic complement, although not necessarily small as virions, do not possess tails and their invasion processes are less well documented. Part II of this thesis is concerned with this problem for the "tail-less" icosahedral bacteriophage ØX174.

For the tail-bearing phages considerable variation exists in the structure of the phage tail (Bradley, 1965). Probably this diversity of structure reflects a similar variation in the molecular detail of the invasion mechanism for these phages, although the main features of the scheme may remain essentially identical. The most detailed description of these events for a tail-bearing phage is

offered by coliphages T2 and T4. The tails of the T-even phages are quite intricate, possessing several morphologically, chemically, and serologically different types of structural components (Brenner, Streisinger, Horne, Champe, Barnett, Benzer & Rees, 1959; Edgar & Lielausis, 1965; King, 1968). The normal tail consists of two concentric hollow tubes, the inner being referred to as the needle or core, and the outer as the sheath. At their distal ends the core and the sheath adjoin the baseplate. Extending from the baseplate are the true attachment organelles of the T-even phages, viz the six long tail fibers that effect the initial stable attachment interaction and also the six short tail 'pins' that are involved in the later, and more intimate attachment of the phage to its host (Simon & Anderson, 1967).

Invasion by T2 or T4 involves then the following steps:

(i) The phage assumes an 'active' state for attachment by extension of the six long tail fibers (Kellenberger, Bolle, Boy de la Tour, Epstein, Franklin, Jerne, Reale-Scafati, Sechaud, Bendet, Goldstein & Lauffer, 1965).

(ii) Stable attachment is established by interaction of the distal tips of the long tail fibers with the receptor sites on the host cell wall; at this stage of the invasion the phage base plate is still more than 1,000 Å from the bacterial surface.

(iii) The phage repositions by altering the conformation of the long

tail fibers which, however, remain attached to the bacterial surface. The rearrangement brings the phage into intimate contact with the cell. Direct interaction between the short tail pins and the exterior portions of the cell has been postulated at this stage of the invasion process (Simon & Anderson, 1967).

(iv) The tail sheath contracts removing the base plate from the bacterial surface while the core penetrates about 120 Å into the cell wall. Viral DNA can now penetrate into the host cell from the phage head via the tail core.

T-even phages are the classic example of penetration by injection of viral nucleic acid; the virion appears as a rather elaborate syringe with the tail core as the needle through which the DNA is injected.

Some interesting and even bizarre variants to this scheme have been elucidated. For example, the DNA of coliphage T5 is injected into the host cell in two distinct phases (Lanni, 1968). The first phase of DNA penetration occurs in the absence of protein synthesis. At this stage of the invasion the phage coats and the unpenetrated DNA (about 92% of the total) can be removed by mechanical shear. The second phase of the injection requires protein synthesis. It has been postulated that the 8% fragment of the T5 genome that penetrates first is a unique sequence of the viral DNA and that it specifies at least two phage-coded functions: (1) Degradation of host DNA, and (2) penetration of

the remaining 92% of the viral DNA. The role of this phage-coded protein that facilitates complete penetration of T5 DNA is unknown.

Another interesting variant is the bacteriophage χ. This phage attacks only flagellated, motile strains of enteric bacteria. The phage apparently attaches to the filament of the flagellum by means of a tail fiber and subsequently moves to the base of the flagellum where the injection event takes place. It has been suggested that the movement of the flagella causes the attached phage to slide along the flagellum to the site for DNA penetration (Schade, Adler & Ris, 1967).

Blue-green algal phage LPP-1 may prove to utilize yet another mode of injection. This phage in purified preparations is reported to have a relatively short tail (Schneider, Diener & Safferman, 1964). However, electron micrographs of algae-attached phage complexes reveal that the virus particles attach to the external walls of the host cell by appendages which appear to be relatively long tails (Smith, Brown, Walne & Goldstein, 1966). This appendage is presumed implicated in the penetration of viral DNA and could prove to be an interesting viral or host organelle.

The tail-less bacteriophages, of relatively small genetic complement, fall into four classes:

(i) The small spherical, male-specific, RNA phages eg. f2, fr, MS2, R17 and Qβ.

(ii) The very large, filamentous, male-specific, DNA phages eg. f1, fd and M13; the viral DNA is both circular and single-stranded.

(iii) The small spherical, DNA phages that contain circular SS DNA eg. ØX174 and S13.

(iv) The lipid-containing, spherical DNA phage that contains circular double-stranded DNA; marine bacteriophage PM2 is the only member of this group, and its invasion mechanism is not yet documented.

The phages of categories (i) and (ii) utilize the F-pili of the host bacteria as receptor sites for phage attachment - the RNA phages attach to the sides of the pilus (Crawford, & Gesteland, 1964) while the filamentous DNA phages attach by their ends to the distal end of the F-pilus (Luria & Darnell, 1967). The phages of category (iii) attach directly to the cell wall of both male and female bacteria (Stouthamer, Daems & Eigner, 1963).

Attachment of phage types (i), (ii) and (iii) may be directly effected by a specific "attachment" protein present as a minor protein species in the mature virion. Conditional lethal mutants are known for the phages of all three groups which produce in the restrictive infection defective virus particles which are incapable of stable attachment to the appropriate host. For the case of ØX174 these defective virions are demonstrated to lack the minor structural protein of the so-called 'spike'

appendages of the phage (Edgell, Hutchison & Sinsheimer, 1969). For the filamentous phages the analogous "attachment" protein has not been identified, although the structural gene is defined (Pratt, Tzagoloff, Erdahl & Henry, 1967); the protein is inferred to be located at one or both ends of the phage particle.

The corresponding protein of the RNA phages has been termed the "maturation" protein. The protein is present as a minor component, probably only one per virion, in the mature phage particle from which it has been purified and characterized (Roberts & Steitz, 1967). The maturation protein is not detected in the defective virus particles produced by amber mutants of R17 cistron A.

It may well be that these three classes of tail-less bacteriophages each utilize a specific viral protein as an attachment organelle. However, in each of the systems discussed, the inability of the defective phage particles to attach to the appropriate host might be a more indirect effect caused by an altered charge or shape of the virion.

The penetration of phage RNA occurs by injection along the F-pilus (Valentine & Wedel, 1965). The phage coat remains attached to the shear sensitive F-pilus after the penetration event (Edgell & Ginoza, 1965). However, it is not yet demonstrated that the viral RNA penetrates the cell by transfer along the inside of the pilus. Viral RNA is however, sensitive at some stage prior to its penetration into the host, to small amounts of extracellular ribonuclease which if

present in the plating medium prevents plaque formation (Zinder, 1963). It has been inferred that the RNase-sensitive stage of the infection occurs while the viral RNA travels along the F-pilus to the cell.

Invasion by the filamentous bacteriophages is poorly defined as yet, however, the preliminary data are quite provocative. As mentioned previously these phages attach by their ends to the distal end of the F-pili of male bacteria (Luria & Darnell, 1967). By analogy with the RNA phages it was felt probable that the viral DNA penetrated the cell via the pilus leaving the phage protein coat attached to the end of the pilus. No transient sensitivity of the infection to extracellular DNase has been reported. Investigations with phage M13 seemed to indicate that only very small amounts of phage protein became associated with the bacteria of an infected culture (Tzagoloff & Pratt, 1964). This was taken as evidence that the protein component of the phage does not enter the host cell and would seem reasonable providing that the majority of the cells were indeed infected in these experiments. However, exactly the opposite conclusion has been drawn from studies with the phage fd (Trenkner, Bonhoeffer & Gierer, 1967). Two lines of evidence are claimed that indicate that phage fd invades the host cell with its protein: (1) The ratio of protein to DNA in infected bacteria is the same as that in the virion, and remains unaffected by various kinds of treatment intended to remove external phage and pili.

(2) There is an efficient transfer of label in parental viral protein to progeny phage. The very intriguing possibility must therefore be entertained that the filamentous phages invade their hosts by an "engulfment" type of mechanism rather than an injection mechanism. Whether the F-pilus plays any role in such a mechanism is not known. The diameter of the fd type phages is about 50 Å ..... about the diameter diameter of a pilus.

Very pertinent to this whole problem is the precise structure of the filamentous phages. One structural model has been proposed based on electron microscopy and X-ray diffraction data on phage fd (Marvin, 1966). It assumes a SS DNA running the 8,000 Å from one end of the virion to the other and back again, with each of the antiparallel DNA strands separately encased in a protein tube. Such a structure would seem to impose limitations on the possible invasion mechanism utilized by the phage. A conventional injection mechanism can be envisioned but it would involve the introduction of a scission in the circular viral DNA molecule and its penetration into the cell as a linear molecule. In fact, penetration of linear fragments of f1 genomes has been observed (Fareed & Valentine, 1968). Since in the cell fd DNA replicates as a double-stranded ring form such a penetration event would demand a mechanism that would recircularize the viral DNA upon its entry into the cell. However, penetration by engulfment of the virion

and intracellular uncoating of the viral DNA would permit the viral DNA to maintain its circular structure. More definitive data concerning the structure of the virions and their mode of penetration are needed to resolve this problem.

The invasion process for bacteriophage ØX174 is the subject of part II of this thesis. Several indirect observations have suggested that ØX174 invades its host by an injection process:

(1) Interaction of ØX174 with cell wall fragments can inactivate the infectivity of the virus and cause the phage DNA to become sensitive to digestion by DNase (Fujimura & Kaesberg, 1962).

(2) Lysates of infected cells contain as well as ØX virions a 70S subviral particle (Sinsheimer, 1959). This particle, referred to as "top component" contains an essentially intact phage coat and a small linear fragment of variable size of the viral DNA (Eigner, Stouthamer, Van der Sluys & Cohen, 1963). The majority of these particles are believed to arise from abortive infections of intact phage with cell debris in the lysates (Sinsheimer, 1968, Bleichrodt & Knijnenberg, 1969).

(3) Dann-Markert, Deutsch and Zillig (1966) described a 57S form of the phage particle that consisted of the phage coat with the viral DNA partially extruded. In the presence of Mg^{++} the 57S form is transformed into a 70S particle that also contains a

full complement of DNA. It was postulated that the ØXDNA of the 57S particle could undergo a near complete, but at least partially reversible, extrusion from the phage coat depending upon the ionic environment.

How the nucleic acid of any phage (other than the T-even phages) traverses the cell wall of the host remains a matter for conjecture. Electron micrographs of cell wall fragments of E. coli C (the usual host for ØX174) indicate the presence of ring-shaped subunits that have an internal diameter roughly the same as the diameter of a phage spike (Tromans & Horne, 1961). The observation generated the speculation that ØX might attach to the cell wall and establish an association between a spike and a subunit of the cell wall, to form an apparatus for DNA transfer.

The results of an investigation into the early stages in the ØX infective process are presented in section II.2. Section II.2.1 elucidates the three distinct early stages in the infective process viz, attachment, eclipse and DNA penetration; the material is presented in the form of a manuscript. Section II. 2.2 consists of some further experiments concerning the penetration of viral DNA under atypical conditions. The final section, II. 2.3, comprises another manuscript and presents data on the kinetics of the attachment and eclipse interactions and the influence of viral mutation on them.

The manuscripts include a detailed discussion of the results contained therein. However, in section II. 3 some more general discussion will be presented together with an overall summary of the results presented in section II. 2.

II. 2 Results

II. 2. 1 The Early Steps in the Infection Process: Attachment, Eclipse, and DNA Penetration

The results in this section have been submitted for publication in the Journal of Molecular Biology. The paper is included in the form in which it was submitted.

Running Title: Early Steps in ØX Infection

SUMMARY

Three distinct stages are demonstrated in the process whereby ØX174 invades its host:

(1) Attachment: The phage attach to the cell in a manner that does not irreversibly alter the phage particles. Phage attachment *per se* does not harm the cell.

(2) Eclipse: The attached phage undergo a conformational change Some of the altered phage particles spontaneously detach from the cell (in a non-infective form) while the remainder are more tightly bound to the cell. The altered phage particles detached (spontaneously or chemically) from such complexes have at least 40% of their DNA extruded from the phage coat. It is proposed that this particle is, or derives from, a direct intermediate in the penetration of the viral DNA.

(3) DNA penetration: A fraction of the attached, eclipsed phage particles corresponding in number to the plaque-forming units complete DNA penetration. The penetrated DNA is found in the cell as RF*, and the empty phage protein coat remains firmly attached to the exterior of the cell. This step is inhibited by prior irradiation of the phage with relatively high doses of UV light.

Both eclipsed phage particles and empty phage protein coats may be dissociated from infected cells; some of their properties are described.

1. INTRODUCTION

The process whereby phage T4 with its highly structured tail invades the host bacterium has been described in detail (Simon & Anderson, 1967). These early steps in the infection process are less well documented for other phage systems. The information available concerning the mechanism by which icosahedral phages, without a tail, introduce their nucleic acid into the host cell derives mainly from investigations on RNA phages. These phages attach to the sides of the F-pili of male bacteria (Crawford & Gesteland, 1964) and their RNA penetrates along the pilus into the host cell (Valentine & Wedel, 1965); the pilus has been inferred to act as a "tail" for the RNA phage. Bacteriophage ØX174, however, a small icosahedral phage that contains a single molecule of SS DNA (Sinsheimer, 1959), attaches directly to the cell wall (Stouthamer, Daems & Eigner, 1963) of both male and female bacteria and only a limited description of its invasion process has been presented.

Fujimura and Kaesberg (1962) observed that the interaction of ØX174 with cell wall fragments could inactivate the infectivity of the virus and cause the phage DNA to become sensitive to digestion by DNase. This observation implied that ØX might penetrate into the host cell by

an injection process which takes place at the phage receptor site. That the host plays some role in this penetration event was reported by Knippers, Salivar, Newbold & Sinsheimer (1969) who found that starved cells, in the starvation medium, do not permit the viral DNA to penetrate the cell. Immediately upon the addition of nutrient medium to such starved cells, DNA penetration ensues. It has also been observed that a fraction of the input virus undergo an abortive eclipse and are spontaneously detached from the cells (Sinsheimer, Starman, Nagler & Guthrie, 1962).

In order to provide a detailed description of the invasion process for ØX174 methods have been devised to recover the input virus particles from the phage-cell complexes for characterization. ØX174 attachment to cells is resistant to both shear and enzymatic digestion (Edgell, personal communication). The attachment has a divalent cation requirement best satisfied by Ca^{++}. A buffer composed of sodium tetraborate and EDTA has been found to elute efficiently the coat protein material of the infecting virus particles from infected complexes throughout the latent period. The level of EDTA used in this treatment is toxic both to uninfected cells and to infective centers and is sufficient to cause the release from the cell of an appreciable quantity of cellular protein. However, the application of the elution procedure to infected cells and the characterization of the virus and

subviral particles recovered from the complexes has elucidated three stages to the invasion process; they are denoted (a) attachment, (b) eclipse and (c) DNA penetration.

2. MATERIALS AND METHODS

(a) Biological

The biological materials and methods used here are those described by Newbold & Sinsheimer (19) with a few additions described below.

(i) E. coli C/ØX is a ØX-resistant strain isolated in this laboratory; the strain is also resistant to the extended host range mutants of ØX.

(ii) E. coli C_1 is a spontaneous mutant derived from E. coli C which is resistant to ØX wild type; this strain is sensitive to the extended host range mutants of ØX. (If a group of ØX-resistant strains is isolated by selection from E. coli C then about 90% are of the C/ØX type and 10% are like C_1 (Hutchison, personal communication). For both strains the ØX-resistance is caused by the inability of the virus to attach to the cells.)

(iii) E. coli THU was described by Stern, Sekiguchi, Barner & Cohen (1964).

(iv) E. coli K12, W6 is a ØX-resistant strain used as the normal host in the spheroplast assay for ØX DNA (Guthrie & Sinsheimer, 1963).

(v) TPG medium is composed of 8.0 g KCl, 0.5 g NaCl, 1.1 g NH_4Cl, 0.2 g $MgCl_2$, 6 H_2O, 0.023 g KH_2PO_4, 0.8 g sodium pyruvate, 11.44 g Trizma HCl (Sigma), 3.32 g Trizma Base (Sigma), 1.0 ml. 1 M $CaCl_2$ and 1.0 ml. 0.16 M Na_2SO_4 per liter of distilled water. The addition of 0.2 ml. of 10% glucose solution to 10 ml. of TPG medium permits full bacterial growth.

(b) Labeled phage and top component

Phage preparations labeled only with ^{32}P were grown using E. coli C as host; all other labeled preparations were grown in E. coli THU.

The several labeled virus preparations were made in TPG medium according to the following protocol. For growing E. coli THU, TPG medium is supplemented with adenine 5 μg/ml., thymine 10 μg/ml., L-histidine 20 μg/ml. and uracil 10 μg/ml.

Cells are grown at 37°C to 2 x 10^8 cells/ml. The cells are collected, resuspended in fresh medium and ØXam3 is added to a multiplicity of about 5. Immediately after the addition of phage the radioactive precursor is added. The infection proceeds at 37°C for 2 to 3 hr. The cells are then harvested by centrifugation and resuspended in 2 ml. 0.05 M borate. 0.3 ml. lysozyme solution (2 mg/ml. 0.05 M borate) and 0.6 ml. 4% EDTA are added in sequence. After 10 min incubation at room temperature the cultures are frozen and thawed three times to complete

the lysis. The lysates are vortexed vigorously to reduce their viscosity and cell debris in the crude lysate is removed by centrifugation. The lysate is layered onto a 25 ml. 5 to 20% sucrose gradient (0.05 M borate) and centrifuged at 5°C in the SW25.1 rotor for 3 hr at 25,000 rev./min. The phage (114S) and top component (70S) fractions are isolated from this gradient and dialysed against borate buffer at 0°C. Phage preparations are further purified by CsCl equilibrium density gradient centrifugation (Sinsheimer et al., 1962); top component is resedimented on another 25 ml. sucrose gradient.

For preparations labeled with ^{3}H-L-histidine slight modification is made to this procedure. The cells (THU) are harvested at about 8×10^{7} cells/ml. and washed once in TPG. They are then resuspended in fresh TPG medium and supplements are added: adenine, 5 μg/ml.; thymine, 10 μg/ml.; L-histidine, 1 μg/ml.; uracil, 10 μg/ml. The cells continue to incubate at 37°C for 3 hr -- a period sufficient to deplete the histidine in the medium. ØXam3 is added to a multiplicity of 5 together with the ^{3}H-L-histidine. The histidine is at a specific activity of 4 to 5 C/mmole; a concentration of 1 μg/ml. L-histidine is sufficient for an adequate yield of phage.

The doubly labeled phage used in Results (b) were made with ^{3}H-L-histidine at 5.15 C/mmole and with 0.1 ^{32}P atom per virus particle.

(c) Chemicals

(i) Thymine-methyl-^{3}H was purchased from New England Nuclear Corporation.

(ii) ^{3}H-L-histidine was purchased from Schwarz Bioresearch Inc.

(iii) Carrier free ^{32}P was obtained from Nuclear Consultants.

(iv) Micrococcal nuclease was purchased from Worthington Biochemicals Corporation.

(v) α-^{32}P-dATP was purchased from International Chemical and Nuclear Corporation; its specific activity was 750 mC/mmole.

(vi) Purified E. coli DNA polymerase and initiator (a DNase I digest of calf thymus DNA) were the generous gifts of Dr. L. Dumas.

(vii) SV40 was obtained from Mr. R. Watson and Dr. J. Vinograd.

(viii) Anti-ØX rabbit serum was prepared by S. G. Krane according to the procedure of Rolfe & Sinsheimer (1965).

(d) The borate-EDTA elution procedure

This procedure is carried out in a cold room at $2^{o}C$.

The complexes are collected by centrifugation and are resuspended in 2 ml. borate-EDTA (0.05 M sodium tetraborate, 6mM EDTA, pH 9.1). The cells are pelleted again and the supernatant is decanted; this supernatant fraction is the first borate-EDTA eluate fraction. The

cells are again resuspended in 2 ml. borate-EDTA and the procedure is repeated as desired.

(e) Other Procedures

(i) DNA is extracted from phage and eclipsed phage particles as described by Guthrie & Sinsheimer (1963).

(ii) Neutral sucrose gradients were 5-20% sucrose in 0.05 M sodium tetraborate. CsCl equilibrium density gradient centrifugation of virus and cell lysates has been described (Sinsheimer et al., 1962).

(iii) TCA-precipitation and counting procedures were previously described (Newbold & Sinsheimer, 1970).

3. RESULTS

(a) Infecting phage particles and progeny intracellular phage are in distinct cellular locations.

Exposure of bacteria to EDTA increases the permeability of the cell to a variety of substances (Leive, 1965 ; Buttin & Kornberg, 1966; Ennis, 1967) and can also induce some leakage of components from the cell (Heppel, 1967; Leive, Shovlin & Mergenhagen, 1968). The concentration of EDTA used in the elution procedure is toxic both to uninfected cells and infective centers. The procedure has also been found to release from infected cells, labeled with ^{3}H-L-histidine, as much as 15% of the cold TCA-precipitable radioactivity. In view of

this finding it was necessary to determine if the procedure could elute progeny intracellular phage from infected cells late in the infection.

E. coli C cells were infected with ^{3}H-L-histidine-labeled ØXam3 to a multiplicity of 4. After 30 min of infection at 37°C the culture was divided into two equal portions and the cells were collected by centrifugation. One of the pellets was resuspended in SVB and the other was washed three times with borate-EDTA and finally resuspended in SVB; both samples were then assayed for radioactivity and intracellular PFU. For both cultures the titer of intracellular progeny phage was identical -- some 400 PFU/cell. However, the borate-EDTA washings eluted 88% of the ^{3}H-label which was found associated with the cells in the control. This result demonstrates that the protein material of the infecting phage and the progeny intracellular phage are confined to different cellular locations and suggests that the protein moiety of the input phage remains attached to some exterior portion of the cell.

(b) The elution procedure applied to cells infected with doubly-labeled (^{32}P, ^{3}H-L-histidine) and synchronized by the starvation procedure.

E. coli C cells were grown in 200 ml. KC broth to 2 x 10^{8} cells/ml. at 37°C. The cells were collected by centrifugation, washed once with 80 ml. SVB, resuspended in 20 ml. SVB and set to incubate

at 37°. After 40 min of starvation the cells were transferred to 15°C. ^{32}P, ^{3}H-L-histidine ØXam3 was added to a final multiplicity of 0.6 PFU/cell and the culture was allowed to incubate at 15°C for 1 hr. The phage-cell complexes were pelleted in the cold and resuspended in 20 ml. of fresh SVB at 15°C. The culture was then subdivided into 3 equal portions. The first portion was left at 15°C and an equal volume of KC broth, also at 15°C, was added; this culture was allowed to incubate at 15°C for 45 min. The other two portions were both set to incubate at 37°C. After 10 min one of the cultures was transferred to an ice bath, never having been exposed to nutrient medium, while to the other culture an equal volume of KC broth was added and the infection was allowed to develop at 37°C for a further 30 min. All 3 cultures were then brought to 0°C. Each of the cultures contained the same total radioactivity, viz. 130,000 ^{3}H cts/min and 198,000 ^{32}P cts/min (80% of the phage attached to the cells during the adsorption period at 15°C).

The three samples were centrifuged in the cold to pellet the cells. (The supernatant fractions contained the radioactivity that was spontaneously detached from the cells.) Each pellet was resuspended in 2 ml. borate-EDTA at 0°C and the elution procedure applied to these 3 samples as described in Materials and Methods. Seven washes were performed on each sample. Finally each pellet was resuspended in 2.0 ml. 0.05 M borate.

The fractionation of the radioactivity throughout this regimen was monitored for each of the 3 cultures by sampling 0.1 ml. of all fractions; the aliquots were added onto filter discs, which were dried and counted. The results of this analysis are given in Table 1. They indicate that 30-35% of both the ^{3}H and ^{32}P radioactivity spontaneously detaches from the cells in the two cultures exposed to 37°C, and is recovered in the supernatant fraction. Spontaneous detachment of only 3% of the radioactivity is found for the phage-cell complexes maintained at 15°C. The results further indicate that in all three cultures the protein label is almost quantitatively eluted from the cells by the borate-EDTA elution procedure. The protein label attached to the cells in the culture maintained at 15°C is also recovered by the elution procedure in fewer washings than in the other two cultures.

For the culture maintained at 15°C and the culture kept in the starvation medium at 37°C, the ^{32}P is also almost quantitatively eluted from the cells. However, in the third culture, exposed to both 37°C and nutrient medium (conditions favorable to productive infection), the nucleic acid label becomes partially resistant to elution from the cells; 20% of the ^{32}P cts/min initially attached to the cells at 15°C remains associated with the cells after the seven borate-EDTA washes.

The data of Table 1 suggest at least three steps in the invasion process of ØX174. The first step is an attachment of phage to the cell

that is characterized by very little spontaneous detachment and relatively easy recovery of all the input viral material from the complex by the elution procedure; this step occurs at 15°C, under which condition the input phage do not go into eclipse (Newbold & Sinsheimer, 1970). The second step is isolated in the infection of starved cells in starvation medium at 37°C. It is characterized by considerable spontaneous detachment and relatively more difficult elution of the remaining input viral material (both protein and DNA); under these conditions, eclipse of the input viral infectivity is observed (Denhardt & Sinsheimer, 1965). The third stage is found in complexes that have been exposed to 37°C and incubated in nutrient medium and is defined by the subsequent resistance of a fraction of the input viral DNA to elution from the infected cells.

(c) What is eluted from infected cells?

The seven borate-EDTA washings for each of the three cultures in the experiment just described were pooled and concentrated by dialysis against dry Sephadex. These samples were mixed with tsχh phage as an infectivity marker and then analyzed by sucrose gradient sedimentation and CsCl equilibrium density gradient centrifugation (Fig. 1). The material eluted from complexes maintained at 15°C (Fig. 1a and 1d) behaved by both criteria as a homogeneous component,

indistinguishable from intact phage. The sample obtained from complexes kept in SVB at 37°C (Fig. 1b and 1e) also behaved in both analyses as a single component labeled with both isotopes. This material sedimented more slowly (approximate sedimentation coefficient, 84S) and more heterogeneously than intact phage. This sample had the same buoyant density in the CsCl gradient as intact phage but exhibited a slight heterogeneity on the less dense edge of the band.

More heterogeneity was displayed by the sample that was obtained by elution from infected cells late in infection (Fig. 1c and 1f). This material also sedimented more slowly than whole phage; some dissociation of the protein and DNA labels was observed. The peak of the ^{32}P radioactivity sedimented at 82S, just ahead of the broader ^{3}H profile at 74S. In the CsCl density gradient, this material displayed two buoyant species. One component, labeled with both isotopes, had the buoyant density of whole phage and density heterogeneity on the less dense edge of the band. The second and minor component was less dense than phage and labeled only with ^{3}H. The very heterogeneous sedimentation analysis of this sample (Fig. 1c) was a composite profile of these two distinct buoyant species. Sucrose gradient sedimentation of each of the two distinct components, isolated from the CsCl density gradient (Fig. 2) demonstrated that the species with the buoyant density of whole phage sedimented heterogeneously at 86S (in a manner

identical to that seen in Fig. 1b), while the less dense component labeled only by ^{3}H-L-histidine sedimented as a relatively sharp band at 72S.

The ratio of ^{32}P to ^{3}H radioactivity for the components displayed in Fig. 1a, b, d, e and 2a, is 1.5 and is identical to that for the initial phage stock; for the component shown in Fig. 2b this ratio has a maximum value of 0.1.

Selective assay of the am3 infectivity, by plating on E. coli HF4714 at 40^{o}C, was undertaken to determine the specific infectivity of these various components. The specific infectivity of the initial phage stock was 0.2. (The particle titer in the stock is determined from the ^{32}P radioactivity.) The specific infectivity of the 114S component in Fig. 1a was also 0.2. However, for the 84S (Fig. 1b), 86S (Fig. 2a) and 72S (Fig. 2b) species the specific infectivity was less than 8×10^{-5}.

The material eluted from infected cells that have been maintained at 15^{o}C thus appears to be indistinguishable from the virus in the original stock. The act of attachment to the cell does not confer an irreversible change upon the phage. Once the complexes have been exposed to a higher temperature and the infectivity of the input virus has gone into eclipse, the input viral material is recovered in an altered form. From infected starved cells, never exposed to nutrient medium, the material is recovered as particles for which the ratio of protein to DNA is the

same as for the initial phage. The buoyant density of the vast majority of these particles is that of intact phage; however, the sedimentation properties are markedly different. These particles, sedimenting more slowly and heterogeneously, are denoted "eclipsed phage particles."

The intracellular events of ØX infection and also the step of viral DNA penetration require the addition of nutrient medium to infected starved cells (Denhardt & Sinsheimer, 1965; Knippers et al., 1969). After this event, the input viral material recovered by elution from the complexes has two forms. The major component is the eclipsed phage particle already described, while the minor component has the superficial properties of an empty phage protein coat.

The eclipsed phage particle is characterized by its sedimentation properties and its inability to infect cells. Its lack of infectivity is readily understood since the particle, isolated by the elution procedure from infected cells, is unable to attach to cells. The distinguishing properties of the eclipsed phage particle could be caused by a change in the composition or conformation of the phage coat or its DNA, or by both. The proportional composition of the particle appears to be unaltered as indicated by the ratio of viral protein to viral DNA and by its buoyant density. Sedimentation of the DNA of eclipsed phage particles on an alkaline sucrose gradient as described by Knippers, Razin, Davis & Sinsheimer (1969) revealed that 55% of the DNA was in

the form of intact rings and the remainder sedimented as linear molecules of, or close to, full size. This result implies that at least 55% of the eclipsed phage particles are composed of a full complement of DNA and hence of viral protein. Since 90% of the material in a preparation of eclipsed phage particles has a buoyant density of 1.4 g/ml., it is concluded that essentially all of the eclipsed phage particles have the full complement of viral protein and DNA and therefore that the eclipsed phage particle has not undergone a change in its composition but rather in its conformation.

(d) What is the viral material that spontaneously detaches?

The supernatant fractions containing the spontaneously detached radioactivity in the experiment described in Results (b) were analyzed by sucrose gradient sedimentation and CsCl equilibrium density gradient centrifugation.

The small amount of material that spontaneously detached from complexes kept at 15°C was indistinguishable from the initial phage. The results for the other two samples, which yielded spontaneous detachment of 30-35% of the material, are given in Fig. 3. They indicate that the spontaneously detached material is a particle similar to the eclipsed phage particle, but with a slightly lower sedimentation coefficient. It is also evident from Fig. 3d that after viral DNA penetration has taken place, no so-called empty phage protein coats are

spontaneously detached from the cells.

The structural similarity of these particles and the eclipsed phage particles and the absence of any spontaneous detachment of empty phage coats emphasizes the intimate association between spontaneous detachment and the conformational change that accompanies the eclipse event.

(e) What remains associated with the cell after the application of the elution procedure?

Lysates were prepared of the three cell suspensions that had been subjected to the borate-EDTA elution procedure, described in Results (b), by treatment with lysozyme-EDTA and subsequent freezing and thawing. Aliquots (corresponding to the material from 10^9 cells) of these samples were then analyzed by CsCl equilibrium density gradient centrifugation. The results of such an analysis at a mean density of 1.37 g/ml. are given in Fig. 4. The analysis at a mean density of 1.71 g/ml. is documented in Fig. 5.

The data demonstrate that the residual input viral material is found associated with the cells in four distinct forms: (i) phage, (ii) eclipsed phage, (iii) phage coats, and (iv) RF DNA. For the lysate derived from the culture kept at $15^{\circ}C$, the residual material was intact phage (Fig. 4a); for the culture maintained in SVB, it was eclipsed

phage (Fig. 4b). (The distinction between intact and eclipsed phage was made on the basis of specific infectivity measurements and the density heterogeneity of the component.) The sample derived from infected cells late in the infection displayed both eclipsed phage particles and phage coats as minor components (Fig. 4c), and free RF DNA as the major component (Fig. 5c). No SS DNA is observed in this raw lysate, as previously reported (Sinsheimer et al., 1962). The amount of ^{32}P found as RF DNA in the cells after the DNA penetration step is approximately 20% of the ^{32}P radioactivity that initially had attached to the cells at 15°C. It has already been mentioned that 20% of the virus particles in the phage stock used for this experiment were PFU. This correlation suggests that only the PFU in a phage stock complete the DNA penetration step.

The quantitation of the radioactivity recovered in the different subviral components in Figs. 1e, 1f, 4 and 5 is presented in Table 2. The total of radioactivity recovered in the eluate fractions together with the radioactivity that remained with the cells is slightly greater before the penetration of viral DNA than afterwards. The difference is accounted for by an increased level of spontaneous detachment in that culture in which DNA penetration took place (see Table 1). With this amendment, the data in Table 2 indicate that the DNA penetration step involves the disappearance of a number of eclipsed phage particles approximately

equal to both the number of empty phage coats generated and the number of parental RF molecules found in the cells.

(f) Characterization of the eclipsed phage particle.

A preliminary characterization of the eclipsed phage particle has demonstrated that it has the same composition as phage, but an altered conformation, defined by an increased frictional coefficient and a slight density heterogeneity. The sedimentation coefficient for different preparations of eclipsed phage particles has been found to vary within the range 60-90S. Its dependence upon the multiplicity of infection, the length of time permitted for eclipse, and other variations in procedure have not been studied. For a given preparation of eclipsed phage, kept in borate-EDTA at $0^{o}C$, the sedimentation coefficient decreases slowly with time. However, the density distribution and the infectivity measurements display very little variation. These observations make it difficult to draw a real distinction between the eclipsed phage particle and the spontaneously detached particle, which differ slightly only in their sedimentation coefficients.

The increased frictional coefficient of these particles suggested that the viral DNA protruded partially from the phage coat as proposed for heated phage particles (Guthrie & Sinsheimer, 1960). This further implied that the eclipsed phage particle may be, or may be derived

from, a direct intermediate in the phage DNA penetration process. The succeeding experiments were undertaken to test this possibility.

Like the heated phage particle, the eclipsed phage particle was found to be infective to spheroplasts and sensitive to digestion by deoxyribonuclease. Both eclipsed phage particles and spontaneously detached particles were as infective to spheroplasts as was their isolated DNA. Because the eclipsed particle was found to be unstable in tris-Mg^{++} buffer, experiments with pancreatic DNase I gave ambiguous results. Digestion with micrococcal nuclease was therefore performed in borate buffer in which the particles are stable.

Micrococcal nuclease was found to be active in borate buffer in the presence of 3 mM $CaCl_2$. Fig. 6 shows the time course of the digestion reaction at 35°C with a preparation of ^{32}P-labeled, 75S eclipsed phage particles. The reaction goes to completion in 5 min and renders 75% of the initial ^{32}P acid soluble. An aliquot of this reaction mixture was diluted into 0.05 M borate, 0.01 M EDTA after 10 min digestion, along with a control sample which had been similarly treated but not exposed to the micrococcal nuclease; both samples were mixed with ^{3}H-L-histidine phage and ^{3}H-L-histidine phage coats, prior to centrifugation in a CsCl equilibrium density gradient (Fig. 7). The digested eclipsed phage particle is a relatively homogeneous buoyant species with a density intermediate between phage and phage coats. The

buoyant density of the digested particle is judged to be slightly greater than the value of 1.333 g/ml. found for a preparation of ^{32}P ØX top component (see Fig. 10). The density-homogeneity of the particles in Fig. 7 suggests that the nuclease resistant SS DNA may be a relatively discrete quantity.

Measurements of the salt dependence of the binding of eclipsed phage particles to DEAE cellulose, performed in 0.05 M borate, also suggest that viral DNA protrudes from the particle. In these experiments the ability of labeled ØX components to bind to the resin, and pellet with it, was compared at 0.1 M and 0.5 M NaCl (Table 3). The eclipsed phage particle mimics ØX SS DNA and not phage in its binding properties to this resin. The distribution of radioisotope label specific to the protein and DNA moieties of the particle shows that the particle behaves as a unit and does not dissoiate into free DNA and coat protein. Further, the portion of the eclipsed phage particle that is responsible for the binding properties is identified as the nuclease digestible DNA.

Further evidence for the partial protrusion of ØX DNA from the eclipsed phage particle derives from its template activity for the purified DNA polymerase of E. coli. Intact phage have no template activity in this reaction. For these experiments a large quantity of eclipsed phage particles with a sedimentation coefficient of 65S was

prepared. Fig. 8 shows the time course of the DNA polymerase reaction at 15°C using eclipsed phage particles as template, a digest of calf thymus DNA as initiator and an excess of deoxyribonucleoside triphosphates; the incorporation of α-^{32}P-dATP was used to monitor the synthesis of DNA. With ØX SS DNA as a template, the in vitro DNA polymerase reaction at 15°C proceeds to a calculated level of one round of synthesis, i.e. as much product DNA is synthesized as was present as template DNA (Mitra, Reichard, Inman, Bertsch & Kornberg, 1967; Newbold, unpublished results). For the reaction documented in Fig. 8, 6.8 µg of eclipsed phage particles were present; this corresponds to 1.7 µg of ØX DNA. The polymerase reaction was complete in 2 hr and 0.7 µg of product was synthesized.

If it is assumed that each of the eclipsed phage particles in the reaction mixture was used as a template, then the result suggests that at least 41% of the ØX DNA is exposed in these particles. Control experiments indicate that the reaction in Fig. 8 was completely dependent upon the addition of eclipsed phage and was not limited by insufficient enzyme, initiator or triphosphates.

The product of the reaction documented in Fig. 8 was analyzed by CsCl equilibrium density gradient centrifugation (Fig. 9); ^{3}H-thymine ØX SS DNA and ^{3}H-L-histidine phage were added as markers. 53% of the TCA-precipitable ^{32}P is found as a single buoyant species with a

density somewhat greater than intact phage. No discrete band of ^{32}P is found in the region of the gradient expected of free DNA. The background of acid-precipitable ^{32}P, most probably from the unused triphosphate, is very high in this gradient, and tends to increase at the higher densities. However, the result indicates that at least half of the DNA synthesized at 15°C, utilizing eclipsed phage particles as template in the polymerase reaction, is stably bound to the eclipsed phage particle.

It is concluded that the structure of the eclipsed phage particle is that of the phage with a large portion of the viral DNA extruded from the phage coat. The density heterogeneity of the particle is not an artifact. If an aliquot of the less dense particles is recentrifuged in a CsCl equilibrium density gradient, then they are found as a sharp band less dense than whole phage. Further, the specific infectivity of these less dense particles, measured in the spheroplast assay, is 0.04 relative to the eclipsed phage particles of density 1.4 g/ml. and suggests that these particles may not contain the full complement of ØX DNA.

(g) Characterization of the empty phage protein coat.

A component described earlier (Fig. 2b) had the superficial properties of an empty ØX protein coat. It was obtained by the elution procedure from infected cells after the viral DNA had penetrated the host.

This particle was less dense than phage, and sedimented as a relatively homogeneous species at 72S. The ratio of protein label to DNA label for the component was much greater than for the initial phage stock.

A sample of ^{3}H-L-histidine "phage coats" was derived in essentially the same manner from a preparation of ^{3}H-L-histidine ØXam3. An aliquot of this sample was mixed with ^{3}H-L-histidine phage, ^{32}P ØX top component, and an optical density amount of a preparation containing both intact SV40 virions and SV40 capsids; this mixture was analyzed by CsCl equilibrium density gradient centrifugation and the result is presented in Fig. 10. The so-called empty phage coats are a less dense and more homogeneous buoyant species than ØX top component. The buoyant density of the empty coats is 1.318 g/ml., which is the value computed for a ØX coat devoid of any DNA.

Empty phage coats are also distinguished from ØX top component by their ability to attach efficiently to cells. This attachment of empty coats to E. coli C is inhibited by anti-ØX serum as is the attachment of whole phage (Rolfe & Sinsheimer, 1965). However, the attachment of empty phage coats displays an extended host range. The coats do not attach to the ØX-resistant strains W6 and C/ØX, but they do attach to E. coli C_1 (which is resistant to ØXwt but is sensitive to the extended host range mutants of ØX).

(h) Eclipse and penetration of UV irradiated phage.

Lytle & Ginoza (1969) reported that penetration of ØX DNA was inhibited by prior irradiation of the phage by high doses of UV light. Our results confirm this finding and demonstrate that the fate of such irradiated phage is to produce an eclipsed phage particle.

The experiment essentially followed the procedure described in Results (b). The input ^{32}P, ^{3}H-L-histidine ØXam3 were irradiated with a germicidal lamp to 8 lethal hits per phage. All of the phage that remained attached to the starved cells at 37°C were converted to eclipsed phage particles. However, both the fraction of empty coats generated and the amount of SS DNA that penetrated the cell and was converted to RF were reduced by the UV irradiation to 5% of the levels found in a control infection with unirradiated phage. The unpenetrated DNA was isolated as 76S eclipsed phage particles.

4. DISCUSSION

The invasion of the cell by ØX174 has been shown to involve at least three separable steps, denoted attachment, eclipse and DNA penetration.

The phage first stably attach to the receptor site on the host cell wall in a manner that does not involve an irreversible change to the phage. Attachment, alone, of ØX does not detectably affect the host (Newbold &

Sinsheimer, 1970). At normal temperatures the attached phage rapidly undergo an irreversible conformational change, with a concomitant loss of their infectivity to cells. This is the step of the infection process designated "eclipse." Intimately associated with the conformational change that defines the eclipse event is the spontaneous detachment from the cell of a substantial fraction of the non-PFU in the phage stock; those phage particles that remain attached to the cell are now more tightly bound to the cell than prior to the eclipse event. In the presence of nutrients, penetration of the viral DNA follows the eclipse event for those phage that are PFU and generates an empty phage protein coat that remains firmly attached to the exterior portions of the cell. The penetrated viral DNA is found in the cell as RF. Those particles incapable of plaque formation retain their DNA and remain tightly bound to the cell.

In an unsynchronized infection at normal temperatures, there is considerable overlap of these three stages among the population of infecting phage. However, the three steps can be isolated utilizing the procedure of starvation synchronization (Denhardt & Sinsheimer, 1965) and the temperature dependence of ØX eclipse. Attachment and eclipse are readily separated at 15°C. At this temperature phage attach to cells but do not lose their infectivity. Eclipse and DNA penetration are distinguished in the infection of starved cells in SVB at 37°C. With

the cells in this condition, the viral DNA is unable to penetrate into the cell. However, immediately upon the addition of nutrient medium to the starved cells, they rapidly regain the condition that permits the viral DNA to penetrate. The role of the host in this step seems not to be the maintenance of an active metabolism, but rather some favorable condition of the cell wall or membrane (Knippers et al., 1969).

The altered conformation of the phage particle while it is attached to the cell wall during the eclipse stage is not characterized. However, the structure of the phage particles that spontaneously detach and of the eclipsed phage particles that are recovered by elution from the cell is that of a phage coat with some of the viral DNA protruded. The exact amount of the viral DNA that protrudes in the eclipsed phage particle has been estimated to be 75% from digestion studies with the micrococcal nuclease and 41% from its template activity in the in vitro DNA polymerase reaction. The heterogeneity in sedimentation of the eclipsed phage particle would suggest that the amount of the DNA that protrudes is not a discrete quantity. However, the homogeneous buoyant density of eclipsed phage particles after digestion with micrococcal nuclease suggests that the digestible DNA is a discrete quantity. These results are not necessarily disparate as conceivably the digestion experiments could measure more than the amount of DNA that was initially protruded; alternatively, the polymerase reaction might underestimate that same quantity.

The results indicate that spontaneous detachment is associated with the conformational change that accompanies the eclipse event. Clearly, the spontaneously detached particles undergo an abortive eclipse and must represent non-PFU in the phage stock. Likewise, the eclipsed phage particles that do not undergo the DNA penetration step must represent non-PFU. Whether these non-PFU are predestined to one or other fate by particular structural defects is not known. Heterogeneity among the phage receptor sites on the host may be involved in these phenomena (Rueckert & Zillig, 1962). The ØX mutant tss6 was shown to undergo a subtle conformational change which resulted in a loss of infectivity of the phage and an increased level of spontaneous detachment (Delgarno & Sinsheimer, 1968). It was also suggested in this report that abortive penetration events might occur with this mutant.

If the eclipsed phage particle is a direct intermediate in the invasion process and is not derived by structural rearrangement from an unstable intermediate, then it would seem likely that the eclipse event involves a partial penetration of the viral DNA into the cell. Whether that DNA truly enters the bacterial cytoplasm, as for the penetration of T5 DNA (Lanni, 1968), cannot be inferred; it is known that at no stage during the invasion process is the infection sensitive to the presence of DNase I.

Also associated with the eclipse event is an altered binding of the phage to the host. Prior to eclipse, it is relatively easier to elute the attached phage particles from the cells than after eclipse occurs. This phenomenon is also found for the empty phage coats. Phage coats attached to cells at 15°C and maintained at that temperature are more easily eluted from cells than an identical culture exposed to 37°C for 10 min (Newbold, unpublished data). This result indicates that the eclipse event for a phage may involve only an interaction between the phage coat and the host.

In agreement with the results of Krane (1966), these studies demonstrate that the fate of the viral protein in the infection is to form a 72S particle. The empty coat has properties similar to, but distinct from, ØX top component. The results do not suggest that any viral protein penetrates into the cell; however, a few protein molecules per PFU could do so and would be undetected in this analysis. Empty phage coats retain the ability to attach efficiently and specifically to cells. This indicates that the sites on the coat involved in the attachment interaction are either undamaged in the invasion process or that redundant sites exist. The extended host range displayed by these coats might be attributable to an altered total charge of the particle. The ØX host range mutants are known to be more positively charged than ØX<u>wt</u> (Hutchison, Edgell & Sinsheimer, 1967) and the empty coat, devoid of the negatively

charged viral DNA, should likewise be more positively charged.

Reaction of phage with anti-ØX serum inhibits the attachment step of the infection (Rolfe & Sinsheimer, 1965). The potential infective center obtained by the attachment of phage to cells at 15°C is not inactivated by incubation with anti-ØX serum (Newbold, unpublished data). The phage may not be accessible to the serum after attachment or, alternatively, the reaction with serum may be ineffective once the phage has attached to the cell, even prior to eclipse.

It has been demonstrated that in the absence of DNA synthesis, penetration of viral DNA can occur to give a free SS DNA molecule associated with the cell (Knippers et al., 1969). The inability of phage exposed to relatively high doses of UV light to undertake this step suggests that perhaps UV-induced bonds link the viral DNA to the phage coat.

The receptor on the host for ØX attachment is composed of lipopolysaccharide (J. A. Cameron, personal communication).. The extraction from the cell of 50% of this material by EDTA (Lieve et al., 1968) may be involved in the mechanism of the elution procedure.

It is perhaps significant that the invasion process for ØX174 is similar to that of the structurally similar polio virus (Hofschneider & Hausen, 1968).

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Footnote

*Abbreviations: RF, replicative form; SS, single-stranded; SVB, starvation buffer; TCA, trichloracetic acid, PFU, plaque-forming units.

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TABLE 1

	Complexes at 15°C		Complexes in SVB		Complexes in KC broth	
	^{3}H	^{32}P	^{3}H	^{32}P	^{3}H	^{32}P
Total	133,800	198,500	133,200	197,600	133,100	197,600
Spontaneously detached	3,841	5,912	40,940	58,820	45,170	67,140
Pellet	130,100	192,800	93,200	139,900	88,000	129,900
Eluate # 1	98,040	147,100	44,100	66,220	43,200	45,080
2	28,380	42,570	27,890	41,910	25,780	27,200
3	2,322	3,483	9,301	14,000	8,910	12,480
4	387	581	4,418	6,640	4,300	4,278
5	129	194	2,430	3,681	3,186	3,240
6	0	0	1,020	1,571	995	1,065
7	0	0	480	700	330	420
Total in washes	129,300	193,900	89,640	134,700	86,700	93,760
Washed cells	760	1,180	2,090	3,141	3,168	39,840

TABLE 2

	Before DNA penetration cts/min		After DNA penetration cts/min	
	^{3}H	^{32}P	^{3}H	^{32}P
Eclipsed phage particles*	90,230	135,640	62,080	91,970
Empty phage coats*	-	-	26,200	-
Parental RF DNA	-	-	-	39,040
Total	90,230	135,640	88,280	131,010

*These totals represent the sum of the radioactivity recovered in the borate-EDTA eluates and that which remained associated with the cells.

TABLE 3

Material	Label	Adsorption to DEAE-cellulose	
		0.05 M. borate 0.1 M NaCl	0.05 M borate 0.5 M NaCl
ØX SS DNA	^{3}H-thymine	+	-
Phage (114S)	^{32}P	-	-
Top component (70S)	^{3}H-L-histidine	-	-
Eclipsed phage	^{3}H-L-histidine & ^{32}P	+	-
Eclipsed phage digested with micrococcal nuclease		-	-

FIGURE LEGENDS

Fig. 1. Sucrose gradient and CsCl density gradient centrifugation of the material eluted from cells infected with ^{32}P, ^{3}H-L-histidine ØXam3.

The seven 2 ml. borate-EDTA eluate fractions were pooled and concentrated by dialysis against dry Sephadex to 6.0 ml. Purified ts χh phage were added as an infectivity marker.

A 2 ml. aliquot was layered onto a 25 ml. 5 to 20% sucrose gradient (0.05 M borate pH 9.2) and centrifuged at 5°C in the SW25.1 rotor at 25,000 rev./min for 3 hr (Fig. 1 (a), (b) and (c)). Ten drop fractions were collected from the bottom of the centrifuge tube into empty sterile shell vials; 0.2 ml. aliquots of each gradient fraction were precipitated with TCA and counted.

A 4 ml. aliquot was mixed with 2.2 g CsCl and the solution was centrifuged at 5°C in the SW50 rotor at 37,000 rev./min for 40 hr (Fig. 1 (d), (d) and (f)). Three drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml borate-EDTA; 0.1 ml. aliquots of each gradient fraction were precipitated with TCA and counted.

—o—o—, ^{3}H cts/min; —•—•—, ^{32}P cts/min.

(a) and (d) - The material eluted from infected cells maintained at 15°C.

(b) and (c) - The material eluted from infected starved cells in SVB at 37°C.

(c) and (f) - The material eluted from complexes at 37°C in KC broth.

The position in the gradients of the peaks of the tsχh phage infectivity marker is indicated by the arrow.

Fig. 2. Sucrose gradient sedimentation of the two buoyant species recovered by elution from infected cells at 37°C in KC broth.

The appropriate fractions of the gradient in Fig. 1(f) were pooled, dialysed against borate buffer and concentrated. Purified tsχh phage were added as an infectivity marker. The samples were sedimented on 25 ml. 5 to 20% sucrose gradients (0.05 M borate) at 5°C in the SW25.1 rotor at 25,000 rev./min for 3 hr. Ten drop fractions were collected from the bottom of the centrifuge tube into empty sterile shell vials; 0.05 ml. aliquots of each gradient fraction were spotted onto Whatman glass filter discs, dried and counted.

—o—o—, ^{3}H cts/min; —●—●—, ^{32}P cts/min.

(a) Fractions 9 to 13 of Fig. 1(f).

(b) Fractions 20 to 24 of Fig. 1(f).

The position in the gradients of the peak of the tsχh phage infectivity marker is indicated by the arrow.

Fig. 3. Sucrose gradient and CsCl density gradient centrifugation of the material that spontaneously detached from cells infected with ^{32}P, ^{3}H-L-histidine ØXam3 at 37°C.

The spontaneously detached material was dialysed against 0.05 M borate and then brought to a volume of 6.0 ml. by dialysis against dry Sephadex. Purified tsχh phage were added as an infectivity marker.

A 3 ml. aliquot was sedimented on a 25 ml. 5 to 20% sucrose gradient at 5°C in the SW25.1 rotor at 25,000 rev./min for 3 hr (Fig.3(a) and (c)). Ten drop fractions were collected from the bottom of the centrifuge tube into empty sterile shell vials; 0.7 ml. aliquots of each gradient fraction were precipitated with TCA and counted.

The remaining 3 ml. aliquot was mixed with 1 ml. borate buffer and 2.2 g CsCl and the solution was centrifuged at 5°C in the SW50 rotor at 37,000 rev./min for 42 hr (Fig. 3(b) and (d)). Three drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate-EDTA; 0.4 ml. aliquots of each gradient fraction were precipitated with TCA and counted.

—o—o—, ^{3}H cts/min; —●—●—, ^{32}P cts/min.

(a) and (b) - The material spontaneously detached from infected starved cells in SVB at 37°C.

(c) and (d) - The material spontaneously detached from infected starved cells in KC broth at 37°C.

The position in the gradients of the peak of the tsχh phage infectivity marker is indicated by the arrow.

Fig. 4. CsCl equilibrium density gradient centrifugation (mean density = 1.37 g/ml.) of the material which remained associated with the cells after the elution treatment.

0.4 ml. of the 3 ml. infected cell lysates was mixed with tsχh phage, borate buffer and CsCl to a mean density of 1.37 g/ml. The solutions were centrifuged at 5°C in the SW50 rotor at 37,000 rev./min for 36 hr. Two drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate-EDTA. 0.01 ml. aliquots were removed from each gradient fraction for infectivity assay and the remainder was precipitated with TCA and counted.

—o—o—, ^{3}H cts/min; —●—●—, ^{32}P cts/min.

(a) Culture at 15°C.

(b) Culture in SVB at 37°C.

(c) Culture in KC broth at 37°C.

The position in the gradients of the peak of the tsχh phage infectivity marker is indicated by the arrow.

Fig. 5. CsCl equilibrium density gradient centrifugation (mean density = 1.71 g/ml.) of the material that remained associated with the cells after the elution treatment.

0.4 ml. of the 3 ml. infected cell lysates was mixed with tsγh SS DNA, borate buffer and CsCl to a mean density of 1.71 g/ml. The solutions were centrifuged at 15°C in the SW50 rotor at 37,000 rev./min for 40 hr. Single drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate-EDTA. 0.4 ml. aliquots were removed from each gradient fraction and precipitated with TCA and counted.

(a) Culture at 15°C.

(b) Culture in SVB at 37°C.

(c) Culture in KC broth at 37°C.

The position in the gradients of the peak of the tsγh SS DNA detected in the spheroplast assay (Guthrie & Sinsheimer, 1963) is indicated by the arrow.

Fig. 6. The time course of the digestion of the DNA in eclipsed phage particles with micrococcal nuclease at 35.5°C.

The reaction mixture consisted of 1.0 ml. of ^{32}P eclipsed phage particles in 0.05 M borate, 0.03 ml. 0.1 M $CaCl_2$ and 10 μg micrococcal nuclease; 0.1 ml. aliquots were removed from the reaction mixture and precipitated with TCA.

Fig. 7. CsCl equilibrium density gradient centrifugation of eclipsed phage particles before and after digestion with micrococcal nuclease.

0.07 ml. of the reaction mixture described in Fig. 6 was diluted into 0.05 M borate, 0.01 M EDTA after 10 min of the digestion. A sample of a similar reaction mixture but not containing micrococcal nuclease was also taken. Each sample was mixed with ^{3}H-L-histidine phage, ^{3}H-L-histidine empty phage coats and CsCl to a mean density of 1.33 g/ml. The samples were centrifuged at 5°C in the SW50 rotor at 37,000 rev./min for 9 hr; the speed was then decreased to 30,000 rev./min and centrifugation was continued for a further 40 hr. Single drop fractions were collected from the bottom of the centrifuge tube directly onto Whatman glass filter discs; the discs were dried and counted.

—o—o—, ^{32}P cts/min; —●—●— , ^{3}H cts/min.

(a) Control reaction mixture -- micrococcal nuclease absent.

(b) Eclipsed phage particles digested with micrococcal nuclease.

Fig. 8. Time course of the *in vitro* DNA polymerase reaction at 15°C utilizing eclipsed phage particles as template.

The reaction mixture consisted of 0.1 ml. buffer (0.1 M potassium phosphate, 0.1 M sodium tetraborate, pH 8.5), 0.05 ml. 0.04 M $MgCl_2$, 6 mM β-mercaptoethanol, 0.05 ml. of a solution that is 1 mM for each of the four deoxyribonucleoside triphosphates, 0.02 ml. of initiator (degraded calf thymus DNA, $A_{260} = 10$), 0.01 ml. *E. coli* DNA polymerase (7.5 μg total protein), 0.02 ml. α-^{32}P-dATP (750mC/

mmole) and 0.05 ml. eclipsed phage particles (6.8×10^{11} particles). 0.02 ml. aliquots of the reaction mixture were precipitated with TCA and counted in a Beckman liquid scintillation counter.

Fig. 9. CsCl equilibrium gradient centrifugation of the product of the in vitro DNA polymerase reaction at 15°C with eclipsed phage particles as template.

After 4 hr of the reaction described in Fig. 8, a 0.02 ml. aliquot was removed into 2.0 ml. 0.05 M borate, 0.04 M EDTA at 0°C. 1.0 ml. of this sample was mixed with ^{3}H-thymine SS DNA, ^{3}H-L-histidine phage, borate-EDTA buffer and CsCl to a mean density of 1.55 g/ml. and the solution was centrifuged at 10°C in the SW50 rotor at 40,000 rev./min min for 40 hr. Four drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate-EDTA; the gradient fractions were precipitated with TCA and counted.

—o—o—, ^{32}P cts/min; —●—●—, ^{3}H cts/min.

Fig. 10. CsCl equilibrium density gradient centrifugation of empty phage protein coats.

^{3}H-L-histidine phage coats were mixed with ^{3}H-L-histidine phage, ^{32}P ØX top component, 1.2 O. D. units (at 260 mμ) of a partially purified preparation of SV40 and CsCl to a mean density of 1.33 g/ml.

The solution was centrifuged at 5°C in the SW50 rotor at 37,000 rev./min for 9 hr; the speed was then decreased to 30,000 rev./min and centrifugation was continued for a further 48 hr. Single drop fractions were collected from the bottom of the centrifuge tube into 0.9 ml. 0.015 M NaCl, 0.0015 M trisodium citrate. The absorbance of the fractions was measured at 260 mμ in a Zeiss spectrophotometer; the fractions were then precipitated with TCA and counted.

—o—o—, ^{32}P cts/min; —●—●—, ^{3}H cts/min.

The positions in the gradient of the peaks of the absorbance of the SV40 virions and SV40 capsids are indicated by the arrows.

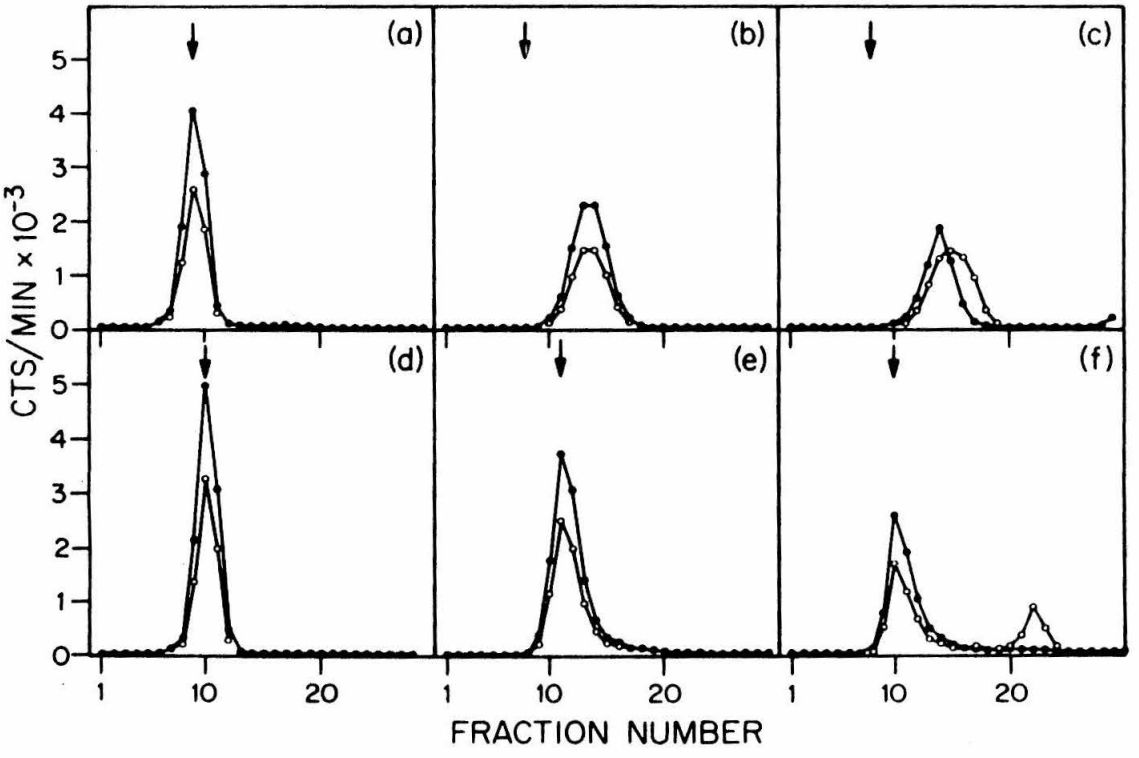

Fig. 1

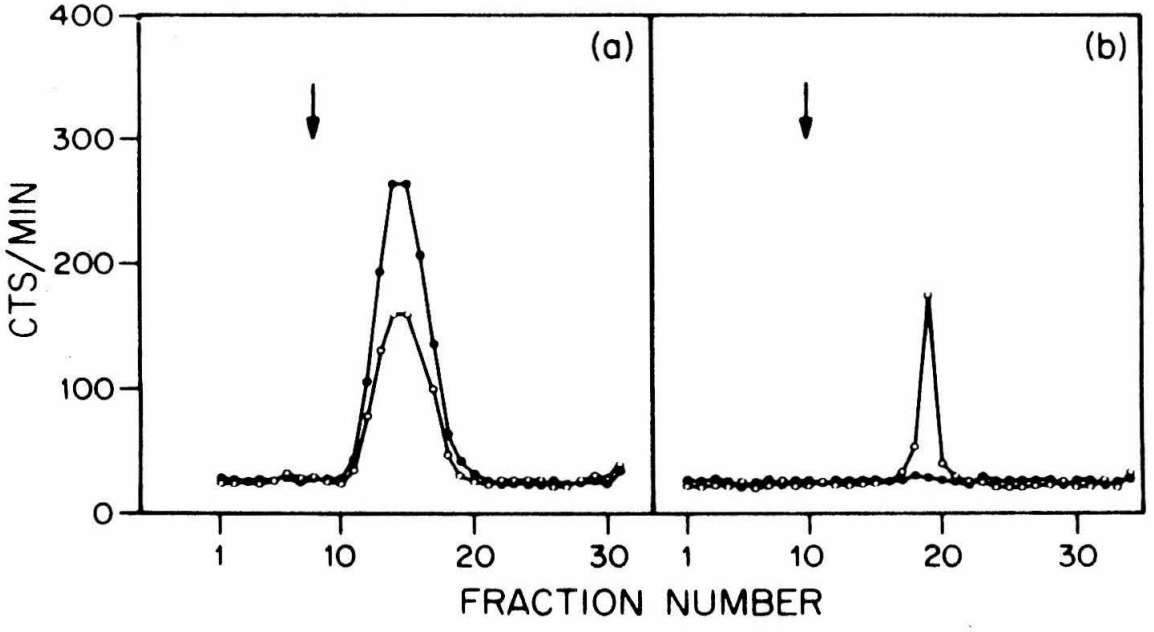

Fig. 2

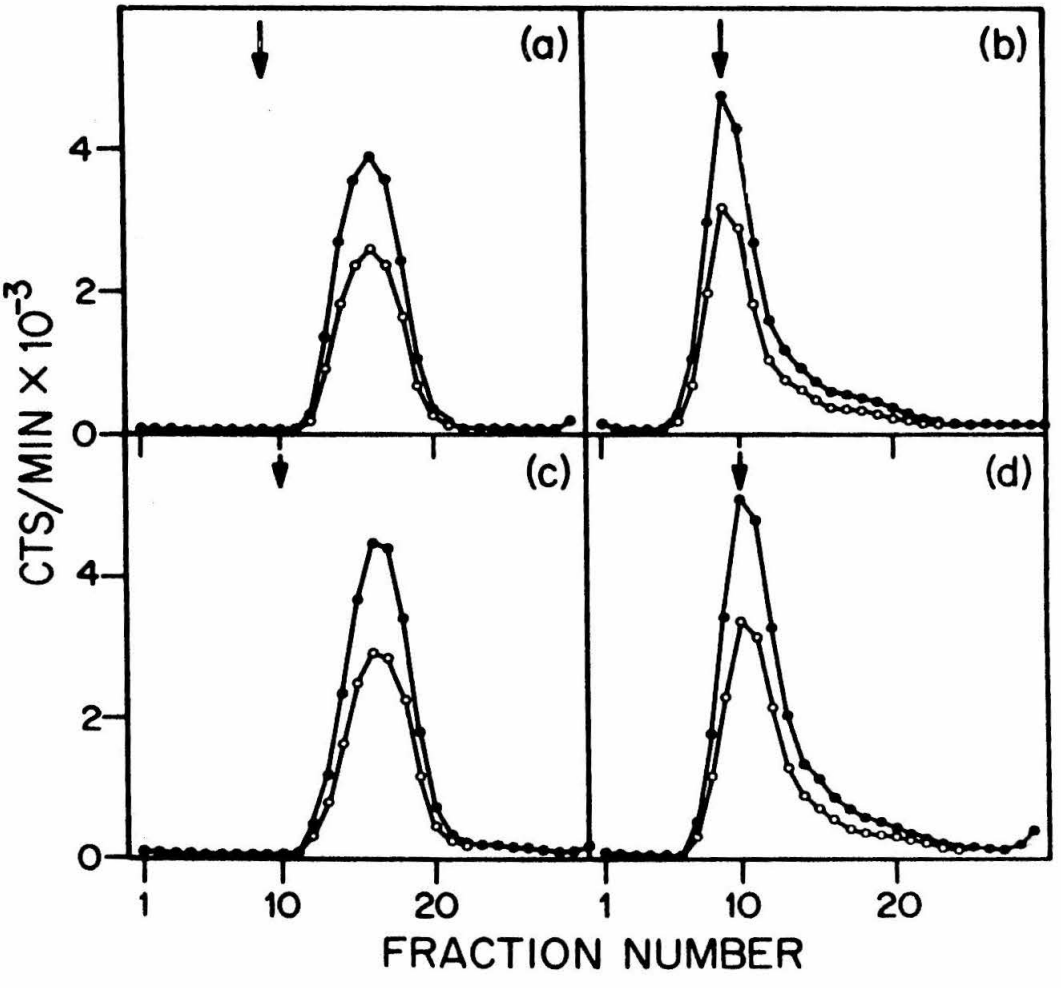

Fig. 3

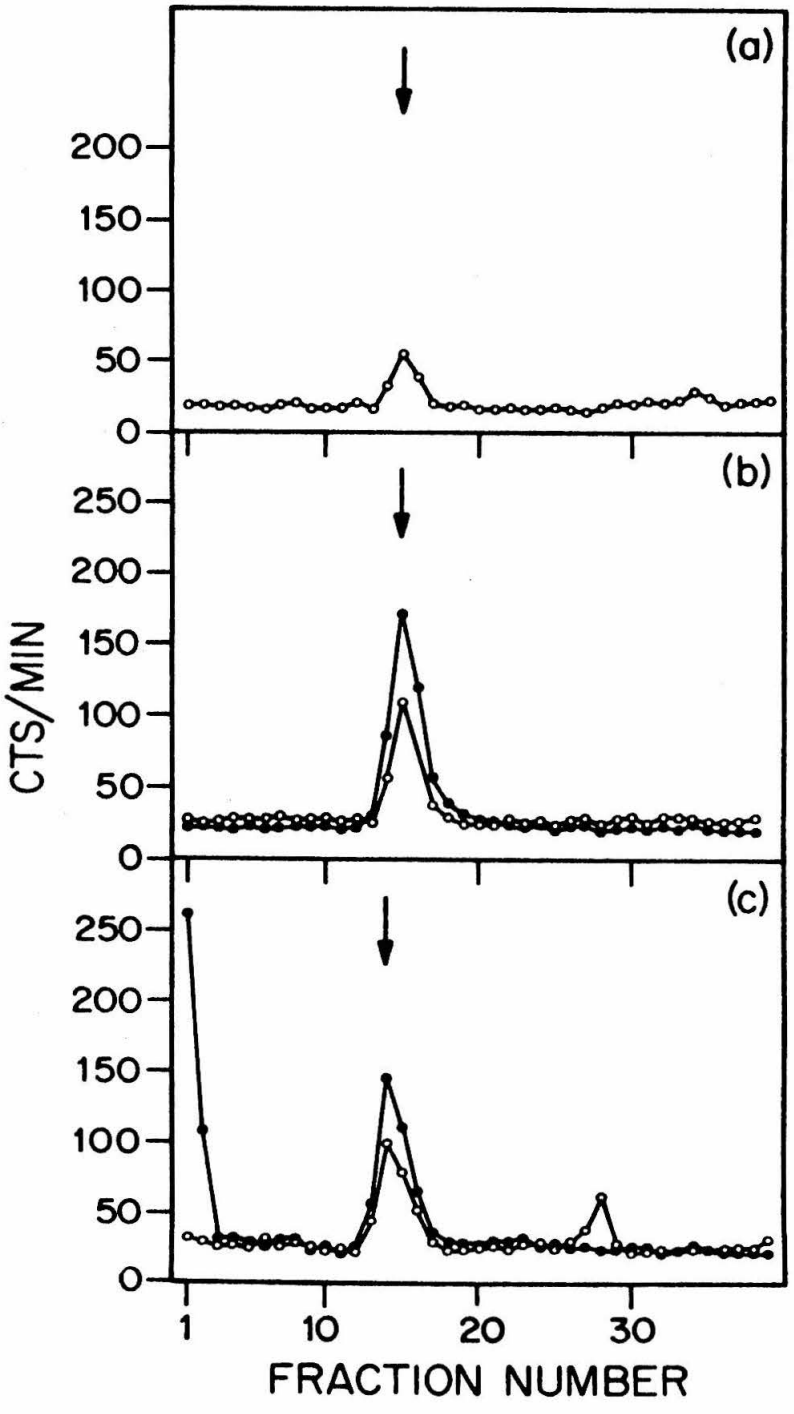
(a)
(b)
(c)
200
150
100
50
0
250
200
150
100
50
0
250
200
150
100
50
0
CTS/MIN
1
10
20
30
FRACTION NUMBER

Fig. 4

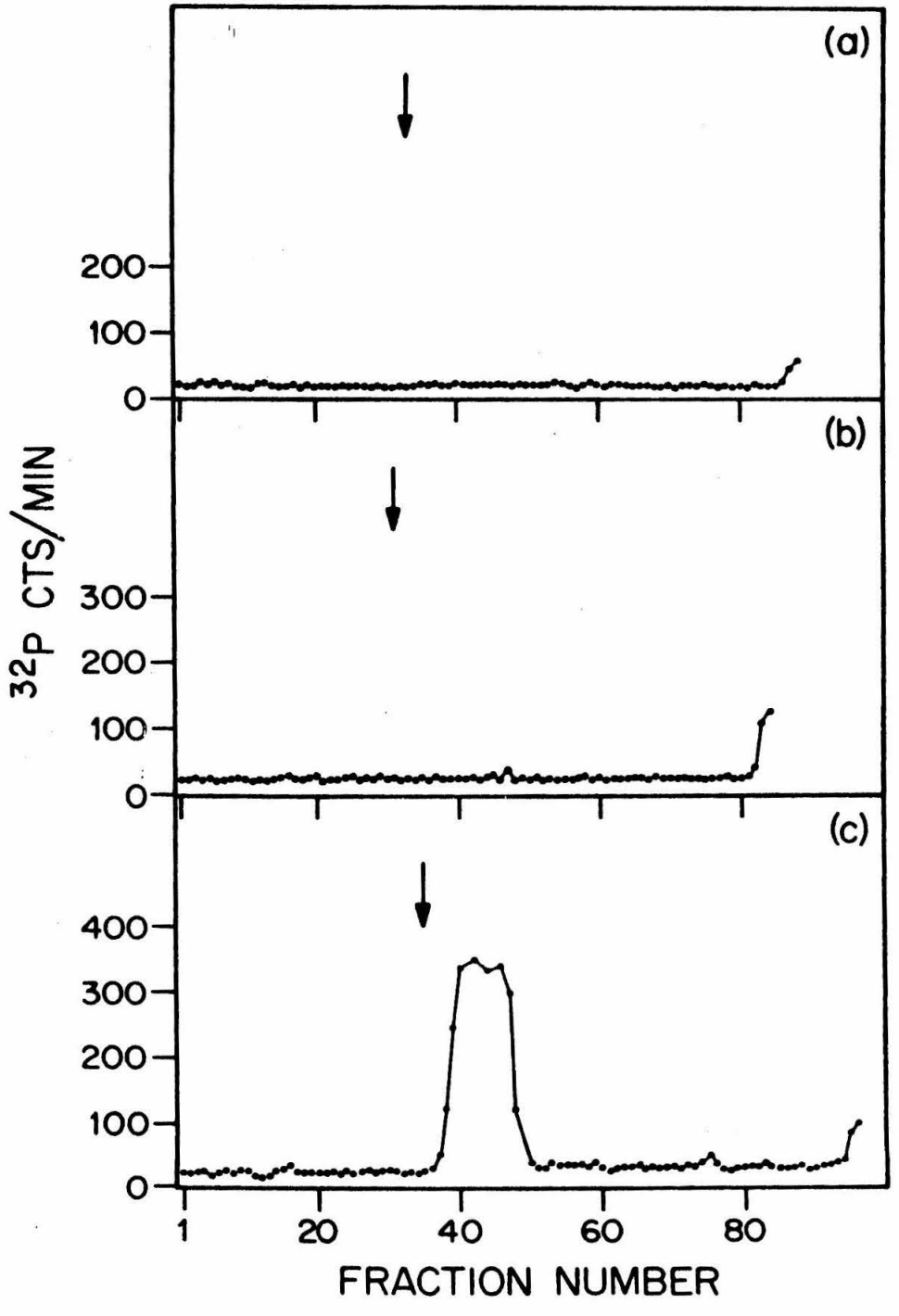
(a)
200
100
0
(b)
300
200
100
0
(c)
400
300
200
100
0
^{32}P CTS/MIN
1
20
40
60
80
FRACTION NUMBER

Fig. 5

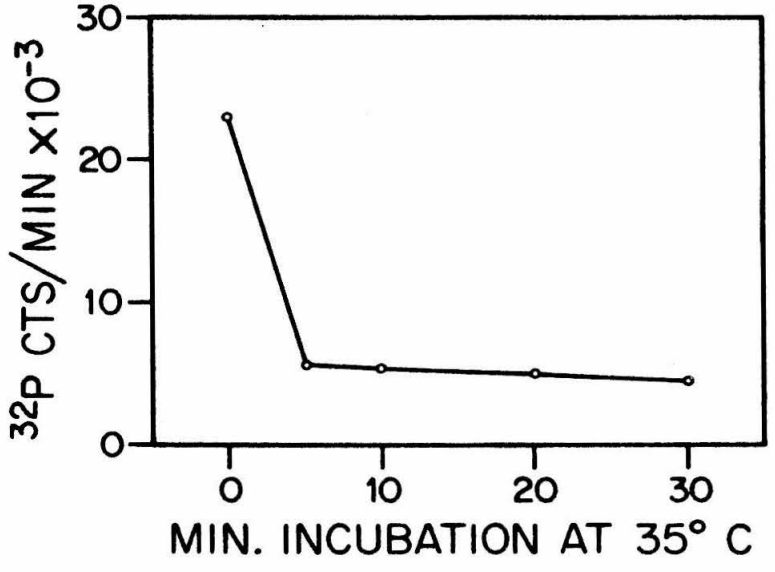

Fig. 6

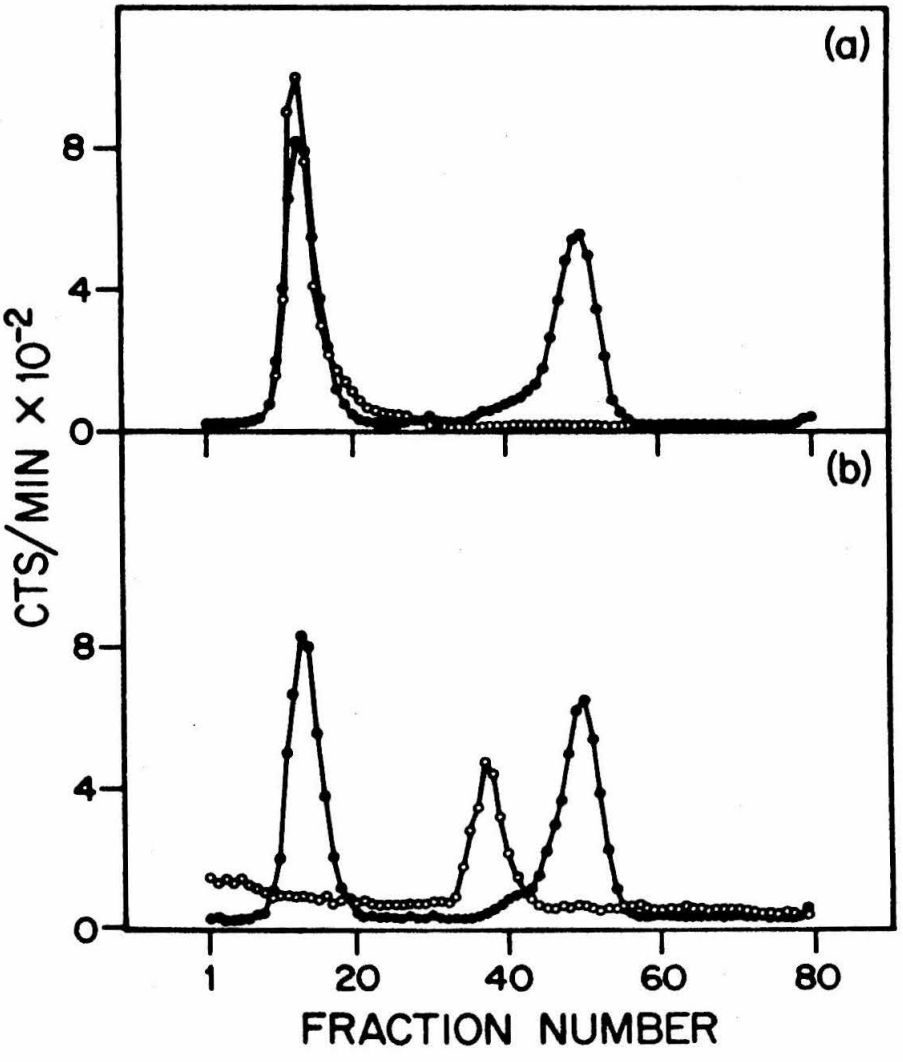

Fig. 7

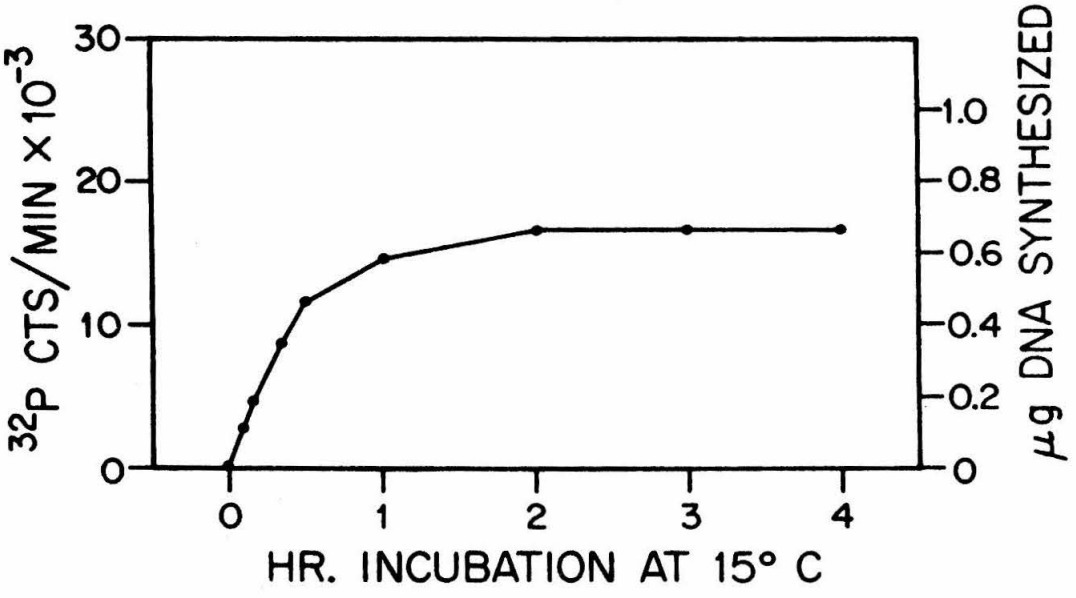
30
20
10
0
^{32}P CTS/MIN X 10^{-3}
1.0
0.8
0.6
0.4
0.2
0
μg DNA SYNTHESIZED
0
1
2
3
4
HR. INCUBATION AT 15° C

Fig. 8

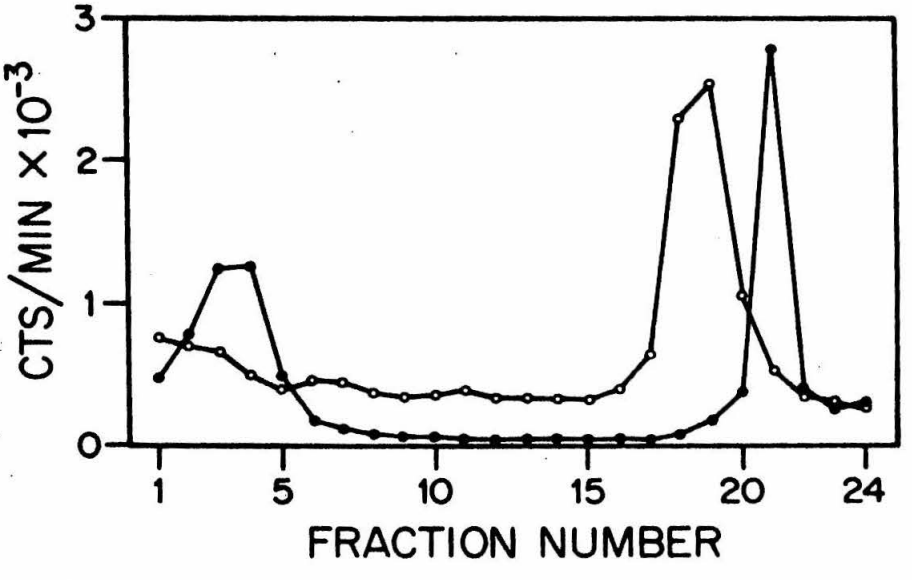

Fig. 9

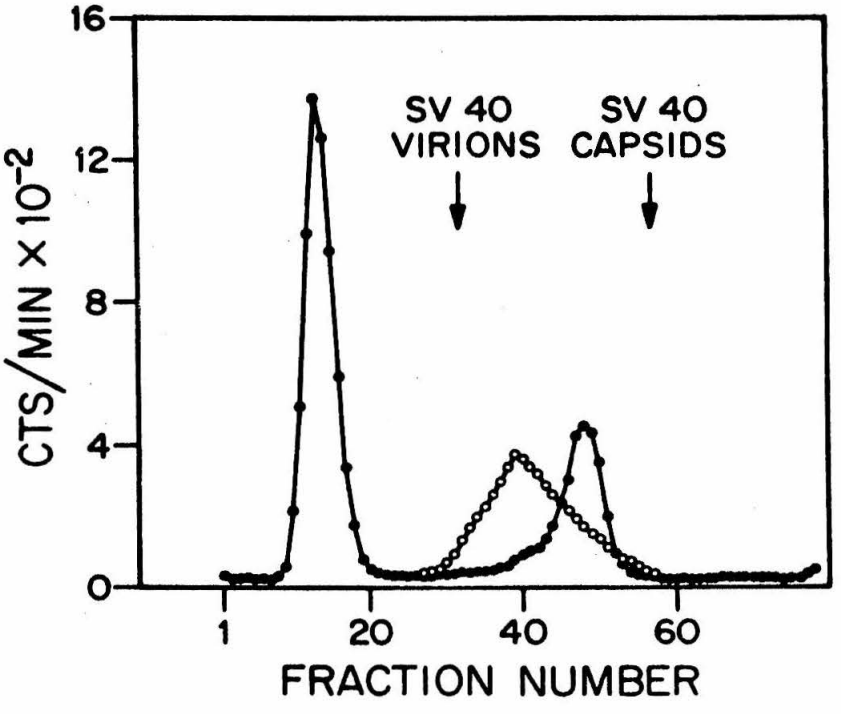
16
12
8
4
0
CTS/MIN X 10^{-2}
SV 40
VIRIONS
SV 40
CAPSIDS
1
20
40
60
FRACTION NUMBER

Fig. 10

II.2.2 ØX DNA Penetration in the Presence of KCN and NaN_3, and for Temporally Excluded Superinfecting Phage.

Similar conclusions to those drawn from the results of these experiments were obtained independently and simultaneously by Dr. Rolf Knippers and have already been published (Knippers, Salivar, Newbold & Sinsheimer, 1969).

The materials and methods employed in these experiments are described in section II.2.1; the double-labeled (^{32}P, ^{3}H-L-histidine) phage are from the same preparation described in that section.

It was mentioned previously that penetration of viral DNA was not effected in the infection of starved cells maintained in the starvation medium (see section II.2.1; Knippers, et al., 1969). Experiments were undertaken to determine if viral DNA could penetrate into cells in which an active metabolism had been arrested by the addition of potassium cyanide (KCN) or sodium azide (NaN_3).

E. coli C cells were grown in 200 ml. KC broth to 2 x 10^8 cells/ml. at 37°C. The cells were collected by centrifugation and resuspended in 40 ml. of fresh broth. The culture was divided into two equal portions; one culture was made 0.01 M for KCN, the other, 0.01 M for NaN_3. After 6 min incubation at 37°C ^{32}P, ^{3}H-L-histidine ØX am3 was added to each culture to a final multiplicity of 2.0 PFU/cell.

Four min after the addition of phage a 9 ml. aliquot was removed from each culture and immediately set at 0^{o}C. After a further 15 min had elapsed a second 9 ml. aliquot was removed from each culture and set at 0^{o}C. The four aliquots were centrifuged in the cold to pellet the cells. The cell pellets were separately resuspended in 2.0 ml. borate-EDTA at 0^{o}C and three cycles of the elution procedure were applied. Finally each pellet was resuspended in 2.0 ml. 0.05 M borate. The three borate-EDTA washings for each of the four samples were pooled and analyzed by CsCl equilibrium density gradient centrifugation (Fig. 1). The samples removed very early in the infection exhibit little or no generation of the empty phage protein coats (Fig. 1a and 1b). However, the later samples indicate their formation in normal yield (Figs. 1c and 1d).

Lysates were prepared of the four cell suspensions that had been subjected to the borate-EDTA elution procedure by treatment with lysozyme – EDTA and subsequent freezing and thawing. Aliquots of these samples were then analyzed by CsCl equilibrium density gradient centrifugation at a mean density of 1.71 g/ml. (Fig. 2). No viral DNA was detected free in the early samples, however, free parental RF was present in the late samples. In an infection under these conditions but in the absence of KCN or NaN_3, very little DNA penetration is found to have taken place during the four min period following the addition of

phage to the cells.

The data demonstrate that the normal DNA penetration process is unimpaired by the presence of either KCN or NaN_3 at 0.01 M — — concentrations sufficient to arrest cellular metabolism and phage replication.

A similar experiment to that just described was undertaken to monitor DNA penetration for temporally excluded superinfecting ØX phage. The DNA of such phage had been previously shown to remain as SS DNA and not be converted to RF (Hutchison & Sinsheimer, 1966). However, these investigations did not indicate if the SS DNA had in fact penetrated the cell.

Again two cultures of E. coli C cells were grown in KC broth at 37^{o}C. One culture was infected simultaneously with a multiplicity of 11 unlabeled am3 and 0.8 ^{32}P, ^{3}H-L-histidine am3. The second culture was first infected with a multiplicity of 11 unlabeled am3 and 14 min later was superinfected with 0.8 double-labeled am3 per cell. Thirty min after initiating the two infected cultures at 37^{o}C the cells were harvested by centrifugation and analyzed as for the previous experiment. The results are presented in Figs. 3 and 4. They indicate that simultaneous infection with labeled and unlabeled phage (the latter at high multiplicity to insure that all the cells are infected) leads to penetration of the labeled viral DNA whereas superinfection with

labeled phage culminates in the generation of no empty phage coats nor the presence of labeled viral DNA in the cells.

Analysis of the labeled viral material eluted from the superinfected cells by sucrose gradient sedimentation revealed that the fate of the superinfecting phage is to form 84 S eclipsed phage particles. Temporally excluded, superinfecting ØX phage appear blocked between the eclipse and DNA penetration stages of the infection.

FIGURE LEGENDS

Fig. 1. CsCl density gradient centrifugation (mean density = 1.37 g/ml.) of the material eluted from cells infected in the presence of KCN or NaN_3 with ^{32}P, ^{3}H-L-histidine ØXam3.

The three 2 ml. borate-EDTA eluate fractions were pooled. Purified tsχh phage were added as an infectivity marker. A 4 ml. aliquot was mixed with 2.2 g CsCl and the solution was centrifuged at 5°C in the SW50 rotor at 37,000 rev./min. for 44 hr. Three drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate EDTA; 0.25 ml. aliquots of each gradient fraction were precipitated with TCA and counted.

—o——o—, ^{3}H cts/min; -●——●-, ^{32}P cts/min.

(a) From infected cells in 0.01 M KCN, 4 min postinfection.

(b) As for (a) but in presence of 0.01 M NaN_3.

(c) As for (a) but 15 min postinfection.

(d) As for (b) but 15 min postinfection.

The position in the gradients of the peaks of the tsχh phage infectivity marker is indicated by the arrow.

Fig. 2. CsCl density gradient centrifugation (mean density = 1.710 g/ml.) of the material which remained associated with the infected cells in the presence of KCN or NaN_3 after the elution treatment.

0.3 ml. of the infected cell lysates was mixed with ts$_{\delta}$h SS DNA, borate buffer and CsCl to a mean density of 1.71 g/ml. The solutions were centrifuged at 15°C in the SW65 rotor at 37,000 rev/min for 41 hr. Single drop fractions were collected from the bottom of the centrifuge tube into 0.5 ml. borate-EDTA. 0.4 ml. aliquots were removed from each gradient fraction and precipitated with TCA and counted.

(a) Culture in 0.01 M KCN, sampled at 4 min.

(b) As for (a) but inhibitor is 0.01 M NaN_3.

(c) As for (a) but time of sampling is 15 min.

(d) As for (b) but time of sampling is 15 min.

The position in the gradients of the peak of the ts$_{\delta}$h SS DNA detected in the spheroplast assay is indicated by the arrow.

Fig. 3. CsCl density gradient centrifugation (mean density = 1.37 g/ml.) of the material eluted from cells infected with both a high multiplicity of unlabeled am3 (m.o i. = 11) and a low multiplicity of ^{32}P, ^{3}H-L-histidine am3 (m.o.i. = 0.8).

The experimental details are essentially identical to those given in the legend to Fig. 1; the cultures were harvested for analysis 30 min. after initiating the primary infection.

(a) The two infections are simultaneous.

(b) The infection with unlabeled phage precedes the superinfection with labeled phage by 14 min.

—O——O— , ^{3}H cts/min;—●——●—, ^{32}P cts/min.

Fig. 4. CsCl density gradient centrifugation (mean density = 1.71 g/ml.) of the material which remained associated with the cells for the experiment described in Fig. 3.

The experimental details are essentially identical to those given in the legend to Fig. 2; the cultures were harvested for analysis 30 min after initiating the primary infection.

(a) The two infections are simultaneous.

(b) The infection with unlabeled phage precedes the super-infection with labeled phage by 14 min.

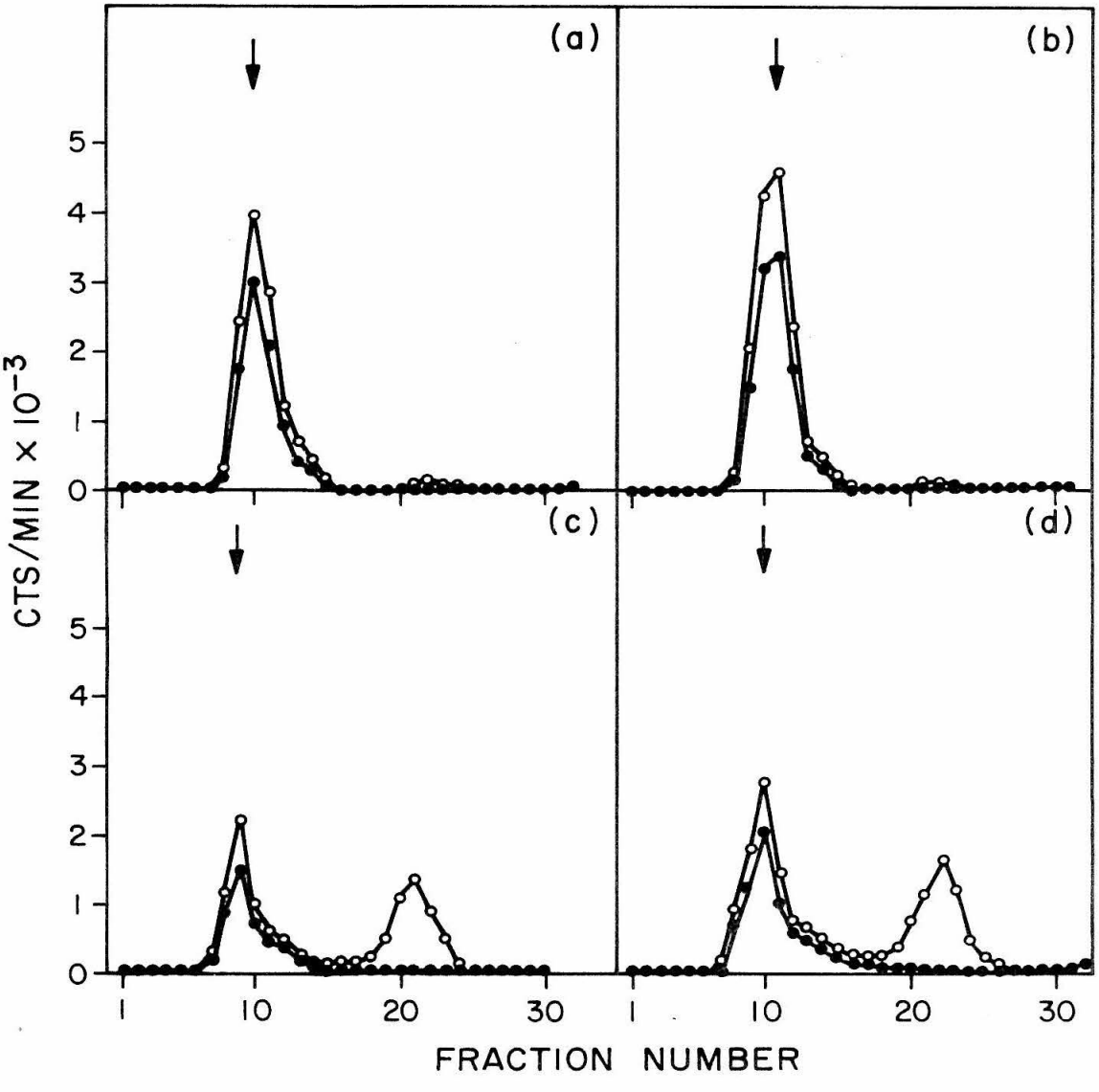

Fig. 1

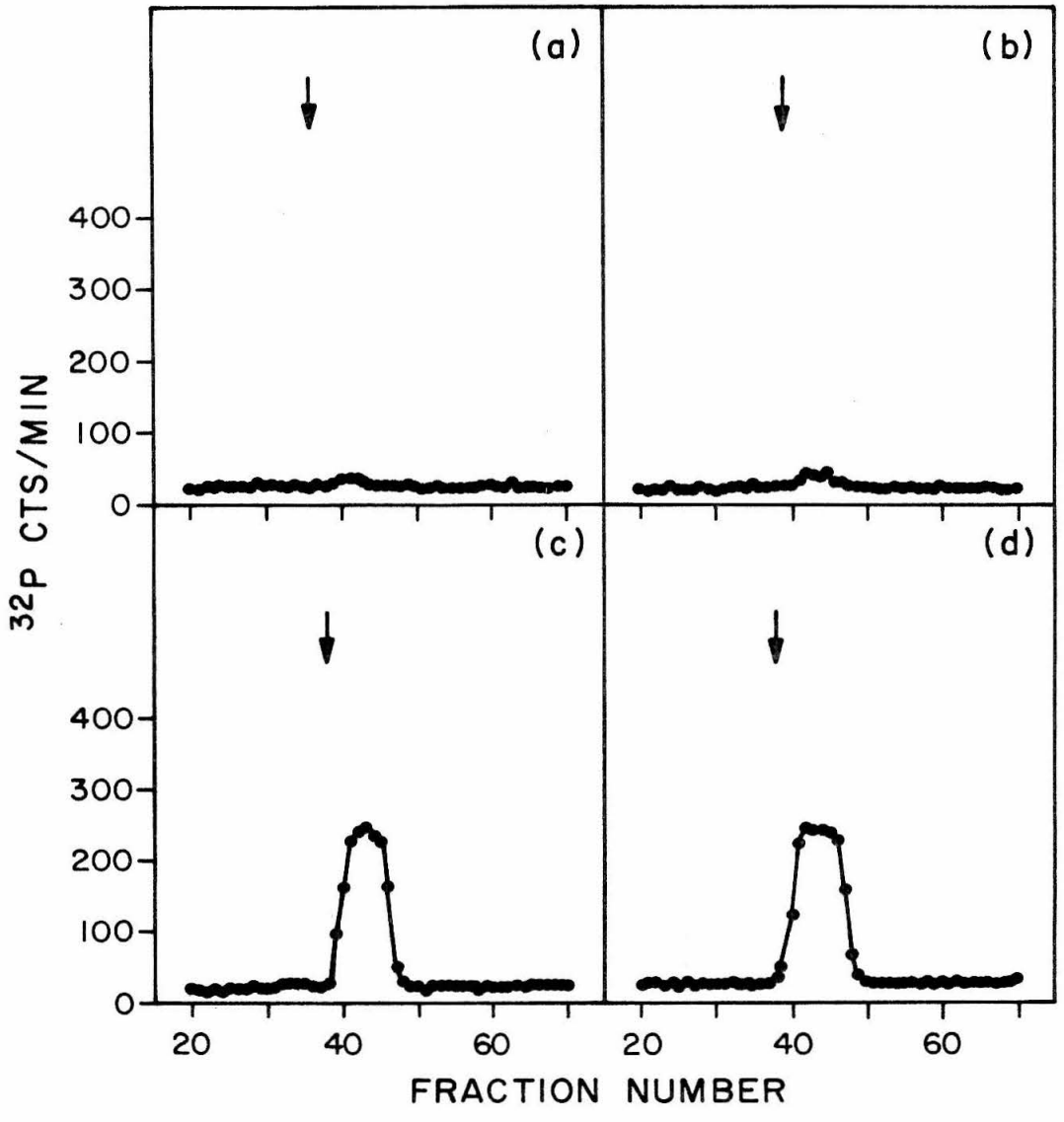

Fig. 2

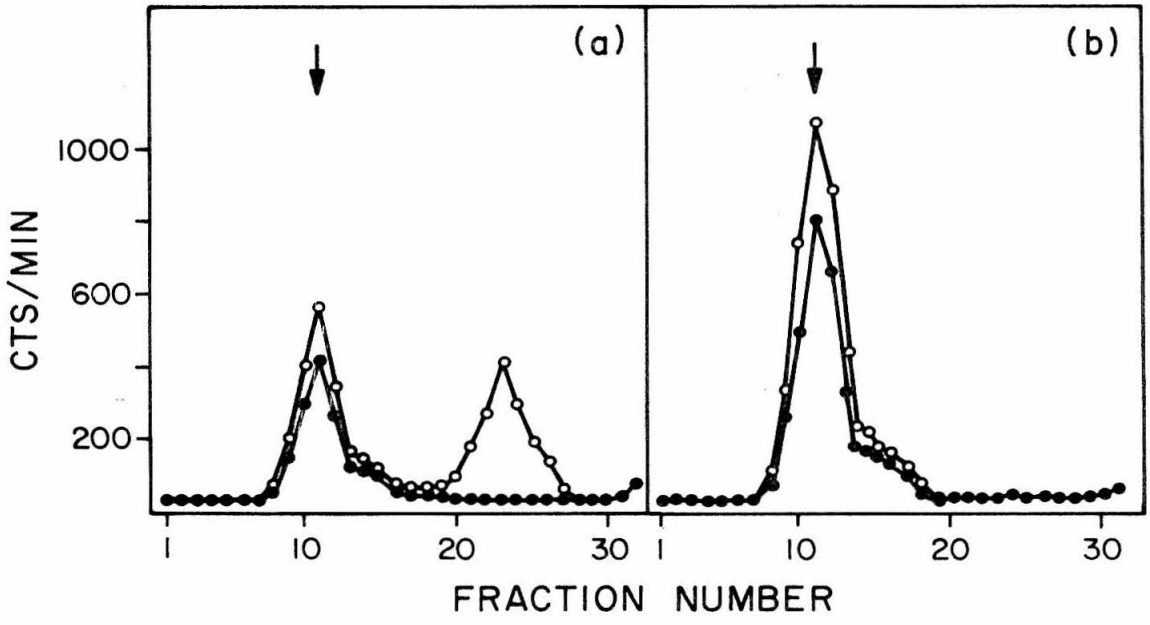

Fig. 3

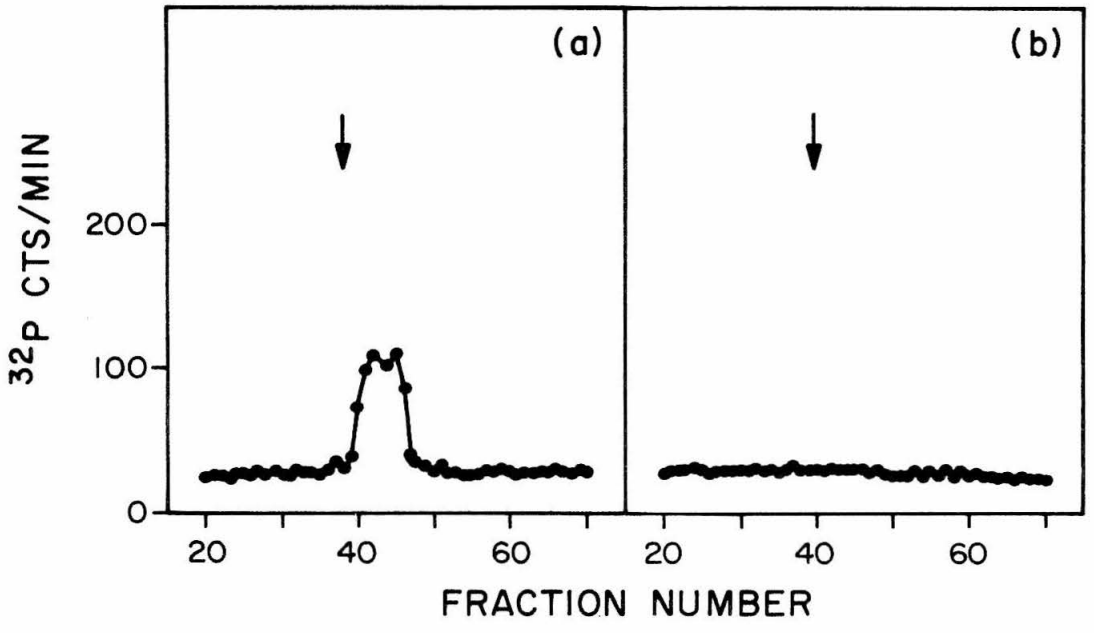

Fig. 4

II. 2. 3 The Kinetics of the Attachment and Eclipse Steps

The results in this section are to be submitted for publication in the Journal of Virology

Running title: Attachment and Eclipse Kinetics of ØX174

ABSTRACT

The products of ØX cistrons II, III and VII are demonstrated to affect the attachment of the phage to its host E coli C; therefore, by inference, these cistrons influence, directly or indirectly, the structure of proteins in the virus particle. Two of the mutations which alter attachment kinetics, ts79 in cistron III and h in cistron VII, affect also the electrophoretic mobility of the virus and emphasize the role of charge in the attachment interaction with the host.

The kinetics for attached phage to go into "eclipse" are first order and biphasic; about 85% of the phage eclipse at one rate ($k = 0.86\ min^{-1}$) and the remainder do so at a distinctly lesser rate ($k = 0.21\ min^{-1}$). No ØX cistrons yet identified affect the eclipse process. The lowest temperature at which eclipse is detected is $19^{o}C$. The Arrhenius activation energy for phage eclipse has the relatively high value of 36.6 kcal/mole indicating the cooperative nature of the event.

INTRODUCTION

In a previous publication in this series three distinct stages were described in the process whereby ØX174 invades the host cell (8). These early stages were denoted attachment, eclipse and DNA penetration. This report presents further data on the attachment and eclipse steps and the influence of viral mutation on these processes.

MATERIALS AND METHODS

Escherichia coli & ØX174 strains. The strains used here have been described previously (7, 8) with the additions listed below.

ØXts4 is a temperature-sensitive mutant assigned to cistron II (coat protein).

ØXts41, ts$_\gamma$ and ts79 are temperature-sensitive mutants assigned to cistron III (coat protein).

ØXts9 is a temperature-sensitive mutant assigned to cistron IV (coat protein).

ØXts41D is a temperature-sensitive mutant assigned to cistron VII (coat protein).

Media. KC broth contains 10 g Bacto Tryptone (Difco), 5 g KCl and 1.0 ml. 1 M $CaCl_2$ per liter of distilled water.

SVB (starvation buffer) contains 5 g KCl, 1 g NaCl, 1.14 g Trizma HCl (Sigma), 0.33 g Trizma Base (Sigma), 0.1 g $MgSO_4$ and

1.0 ml. 1 M $CaCl_2$ per liter of distilled water. This is essentially the recipe described by Denhardt and Sinsheimer (1).

Biological assays. The plating procedures and the preparation of genetically pure mutant phage stocks have been described (6).

Radioactive phage. The preparation of am3 labeled either with ^{32}P or ^{3}H-L-histidine has been described (8).

^{32}P-labeled 110 S am3ts4 defective particles were prepared by a slight modification of the same procedure. The E. coli C cells were infected at 40°C and the culture was incubated at that temperature for 3 hr. The culture was then poured into three volumes SVB at 0°C and the cells were harvested by centrifugation at 2°C. The cell pellet was resuspended in cold 0.05 M borate and lysed at 0°C. The ^{32}P-labeled 110 S species was purified by two cycles of sucrose gradient centrifugation. The purified 110 S species contained 0.1 ^{32}P atom per particle; the specific infectivity of the preparation, calculated from the radioactivity and the titer of plaque forming units was 2×10^{-4} plaque formers/particle, i.e. the level of contamination by infective 114 S phage particles was at most 0.02%.

Attachment kinetics. E. coli C cells are grown in KC broth to 5×10^7 cells/ml. The cells are collected by centrifugation, washed once with one volume of SVB, and then resuspended in two volumes of SVB, i.e. cells are 2.5×10^7 cells/ml. The phage are diluted to

10^8/ml. in SVB. 0.5 ml. portions of each phage preparation are set at 37°C and 9.0 ml. of the cells, prewarmed to 37°C, are added to each to initiate the attachment reaction. Attachment is monitored after 0, 0.5, 1, 2, 4, 8, 15 and 30 min by removing a 0.1 ml. sample from the reaction mixture, diluting it 100-fold into KC broth at 0°C and again 10-fold into a centrifuge tube containing 10^9 E. coli K12 W6 cells (as carrier) also in KC broth at 0°C (the 10^3-fold dilution is sufficient to stop further attachment). When all the samples are prepared, they are centrifuged at 2°C to pellet the cells and the supernatants are titered for residual phage.

Phage are not inactivated in a 30 min period under the conditions of the attachment reaction in the absence of cells.

Attachment of capsids and spikes. The cells are grown in KC broth to 2 x 10^8 cells/ml. Five ml. of these cells are set at 37°C in a centrifuge tube and the radioactive capsids or spikes are added in 0.1 ml. or less. The mixture is incubated at 37°C for 10 min. A 2 ml. portion is removed for assay of total radioactivity and the remaining 3 ml. is centrifuged to pellet the cells. The supernatant is removed and the pellet is resuspended in 3.0 ml. fresh KC broth. The supernatant fraction is added to a previously prepared pellet containing the original number of uninfected E. coli C cells, viz 6 x 10^8 cells; this pellet is resuspended.

Both pellet and supernatant fractions are sampled (2 ml.) for assay of radioactivity. The three 2 ml. aliquots (total, supernatant and pellet) are mixed with an equal volume of cold 12% trichloracetic acid and allowed to stand at 0^{o}C for 1 hr. The precipitate is collected by filtration through a Whatman glass filter disc, rinsed with 10 ml. cold 2% trichloracetic acid, dried and counted. The filter discs are placed into 10 ml. of a toluene liquifluor scintillator (New England Nuclear Corp.) and counted in a Beckman liquid scintillation counter. (The addition of the cells to the supernatant fraction is necessary to obtain equal quenching of radioactivity in the three samples.)

Eclipse kinetics. E. coli C cells are grown in KC broth to 2×10^{8} cells/ml. The cells are collected by centrifugation and washed once with an equal volume of SVB. The cells are then resuspended in 0.1 volume SVB (cells are about 2×10^{9} cells/ml.) and set at 15^{o}C. Phage are added to a multiplicity of about one, and 30 min are allowed for phage attachment at 15^{o}C. The cell-attached phage complexes are collected by centrifugation and resuspended in fresh SVB at 0^{o}C. 0.1 ml. of this culture is diluted into 10 ml. SVB which is equilibrated to the desired temperature; this initiates the eclipse process. Eclipse is monitored by cooling 0.1 ml. samples of the reaction mixture to 0^{o}C by dilution into 5.0 ml. 0.05 M sodium tetraborate, 6 mM EDTA, saturated with chloroform at 0^{o}C.

Further eclipse is prevented by the low temperature (8). The uneclipsed phage are eluted from the cells by the borate-EDTA (8); the chloroform and the borate-EDTA kill the infected cells. The titer of phage in the borate-EDTA solution thus represents the residual, uneclipsed phage.

Under the conditions of the eclipse experiments no inactivation is found for free phage, i.e. no inactivation occurs in a 30 min period at 37^{o}C in SVB.

RESULTS

Kinetics of phage attachment to cell. The time course of phage attachment to E. coli C cells exhibits first order kinetics with respect to phage concentration and the rate constant for ØX wt is 8.2×10^{-9} ml.-min^{-1}-$bacterium^{-1}$. Under these same conditions the lysis defective mutant, am3, demonstrates identical attachment kinetics as the wt.

Mixtures of am3 with each of four different ØX ts mutants were constructed and the relative attachment kinetics of the two phage types in each mixture determined by selective plating for the two phenotypes. (am mutants are assayed on E. coli HF4714 cells at 40^{o}C; ts mutants are plated on E. coli C at 30^{o}C.)(Table 1). The mutants tsX and ts9 have rate constants not significantly different from that of wt and

am3. However, ts79 attaches more slowly to E. coli C than does am3, while tsλh attaches more rapidly. Since tsλ attaches at the wild type rate the more rapid attachment of tsλh can be ascribed to the h (extended host range) mutation.

The ØX mutants ts79 and h have been previously assigned to ØX cistrons III and VII respectively (Hutchison, Ph. D. Thesis, California Institute of Technology, 1969); both these cistrons code for proteins present in the mature phage (5).

Defective attachment of 110 S ts4 particles. The defective 110 S phage particle made in cells infected with ts4 at 40°C has been reported earlier to be defective in adsorption (10). At that time the term "adsorption" was used rather loosely and it often encompassed all of the events in the invasion of the host.

A preparation of ^{32}P-labeled 110 S am3ts4 defective phage was found to be essentially incapable of stable attachment to E. coli C. Ten min was allowed for attachment to the cells (2×10^8 cells/ml.) in KC broth at 37°C. The mixture was then centrifuged to pellet the cells and the supernatant and pellet fractions were assayed for radioactivity. Only 3% of the input ^{32}P was found associated with the cells while 102% remained free in the supernatant. Under these same conditions, at least 80% of a preparation of ^{32}P-labeled (114 S) am3 attaches stably to the cells and pellets with them.

The mutant ts4 is assigned to ØX cistron II (10) which specifies a structural protein of the phage (3). Two of the three cistrons which have been shown here to affect the attachment of phage to the host, viz ØX cistrons II and III are known to code for proteins which are present in the twelve "spikes" of the mature phage (3). The location of the protein specified by cistron VII is uncertain. If the spikes are viral organelles for the specific attachment of the phage to the receptor sites on the host, it might be expected that capsids devoid of spikes would be incapable of specific attachment to cells and further that 'free' spikes might retain that property.

To test this possibility capsids devoid of spikes and free spikes were isolated from a preparation of am3 , labeled with ^{3}H-L-histidine by the procedure previously described (3), and the ability of these labeled subviral components to attach to cells was determined. Capsids attached to E. coli C as efficiently as did intact phage; however capsids also attached efficiently to E. coli C/ØX although they did not attach to E. coli K12 W6 (intact phage does not attach to either of these strains). This attachment of capsids to cells appears, however, to be unrelated to normal ØX attachment: Prior reaction of the capsids with anti-ØX serum does not block their subsequent attachment to cells; prior infection of the cells with saturating levels of non-radioactive virus (50,000 particles/cell) also does not impair capsid attachment. Either

of these reactions inhibits attachment of phage to cells.

The free spikes also attached to cells at a low rate but displayed no host range specificity; they attached to both W6 and C/ØX as well as to E. coli C. It is concluded that the attachment of free spikes and capsids to cells is not closely related to that found for intact phage.

Eclipse kinetics for attached phage. It was reported earlier that at 15°C ØX can attach stably to cells but does not go into eclipse (8), i.e. the phage recovered from such complexes has the same specific infectivity and physical properties as the input virus. The eclipse event which occurs at higher temperatures is defined by a loss of recoverable phage infectivity and a conformational change of the phage (8). The kinetics of the eclipse event are determined by isolating cell-attached phage complexes at 15°C, raising the temperature and monitoring the decrease of recoverable phage infectivity.

Figure 1 shows the eclipse kinetics for am3 at 37.5°C following the protocol described in Materials and Methods (am3 shows the same eclipse kinetics as wt). The kinetics of the eclipse process are first order with respect to phage concentration -- about 85% of the virus particles eclipse at one rate ($k = 0.86\ min^{-1}$); the remainder do so at a distinctly lesser rate ($k = 0.21\ min^{-1}$).

Figure 2 documents the temperature dependence of the eclipse

process (for its faster component). Eclipse was not detected at 16°C or below; it was detected at 19.0°C. The slope of the ln k vs. temperature^{-1} plot corresponds to an Arrhenius activation energy of 36.6 kcal/mole.

Eclipse kinetics for ØXts mutants. Mixtures of am3 and each of the following ØX ts mutants were constructed: ts4 (cistron II), ts41 (cistron III), ts79 (cistron III), tsγ (cistron III), ts9 (cistron IV), ts41D (cistron VII) and tsγh (cistrons III and VII, respectively). By selective plating for the am or ts phenotype the initial eclipse kinetics of both the ts mutant and the reference am3 were monitored in each phage mixture at 37°C. No significant differences in the initial rate of eclipse could be detected among the mutants tested.

DISCUSSION

The rate constant for the stable attachment of ØX wt to E. coli C cells in SVB at 37°C presented in this report -- 8.2×10^{-9} ml.-min^{-1}-bacterium^{-1} -- is in good agreement with the value 6.0×10^{-9} ml.-min^{-1}-bacterium^{-1} determined by Fujimura and Kaesberg (4) to be the optimal rate in simple salt solution (0.1 M $CaCl_2$).

The mutations ts4, ts79 and h in ØX cistrons II, III and VII respectively are demonstrated to affect the attachment reaction. These three cistrons code for structural proteins of the mature virus

(Hutchison, Ph.D. Thesis, California Institute of Technology, 1969). At present four distinct polypeptides are recognized to be present in the mature virus (3; Burgess and Denhardt, personal communication). Three polypeptides are found in the phage spikes, and only one is found in the capsid (3). ØX cistrons II and III code for spike proteins while cistron VII is thought to specify the capsid protein (3; Burgess and Denhardt, personal communication). The remaining structural (spike) protein is tentatively assigned to ØX cistron IV.

The defective 110 S particle produced in ts4 infected cells at 40°C has been shown to lack one spike protein completely (3). The cistron II protein might be directly involved in the attachment of the phage to the bacterial receptor site, in which case the inability of the ts4 defective particle to attach to cells is readily understood. Alternatively the inability of the defective particle to attach to cells might be a more indirect effect caused by an altered charge or shape of the particle.

The two other "attachment mutants," ts79 and h are distinguished from wt by altered electrophoretic mobilities as well (5). The more positively charged the virus, the more rapidly it attaches to the host. (Under the usual conditions of infection both the phage and the host are negatively charged.) In fact, as shown in Table 2 the product of the attachment rate constant and the R_F value (taken from the electrophoresis data of Hutchison, Edgell and Sinsheimer (5)) is approximately

constant for the different phage mutants. These data indicate that the total electrostatic charge on the virus determines, importantly, the rate at which stable phage-cell complexes are formed.

The biphasic kinetics for the eclipse of attached phage particles (Fig. 1) were described earlier (9); as proposed in that report the phenomenon might reflect heterogeneity among the receptor sites of the host. The activation energy for the eclipse process, 36.6 kcal/mole, is quite high . Most chemical and enzymatic reactions have activation energies in the range 11 to 18 kcal/mole. The high value indicates that the eclipse reaction is cooperative in nature. The eclipse event has already been documented to involve a conformational change in the phage protein coat (8).

The ØX mutant cs70 was demonstrated by Dowell to have an abnormal eclipse pattern. This cold-sensitive mutant could attach to the host, but not eclipse, at temperatures of normal eclipse for wt (2). The mutant cs70 remains at present unassigned to a particular ØX cistron. Analyses of a large sample of the available ØX ts mutants of known cistron assignment for aberrant eclipse kinetics yielded no additional examples. As this sample included the electrophoretic mobility mutants ts79 and ts$_{\delta}$h, charge may not play an important role in the eclipse event.

It is of interest that the low temperature threshold for phage

eclipse, about 17°C, is the same threshold as that determined for the maturation of infectious progeny phage and for single-stranded DNA synthesis (7). It is conceivable that the cooperative rearrangement of the same viral protein or group of proteins is involved in all three processes.

ACKNOWLEDGMENTS

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TABLE I

Rate constant for the stable attachment of ØX strains to E. coli C

Phage strain	Rate constant for attachment ($ml\text{-}min^{-1}\text{-}bacterium^{-1} \times 10^{9}$)
am3	9.2
ts γ	8.5
am3	8.2
ts9	8.2
am3	9.0
ts79	5.1
am3	8.0
ts γh	11.3

TABLE 2

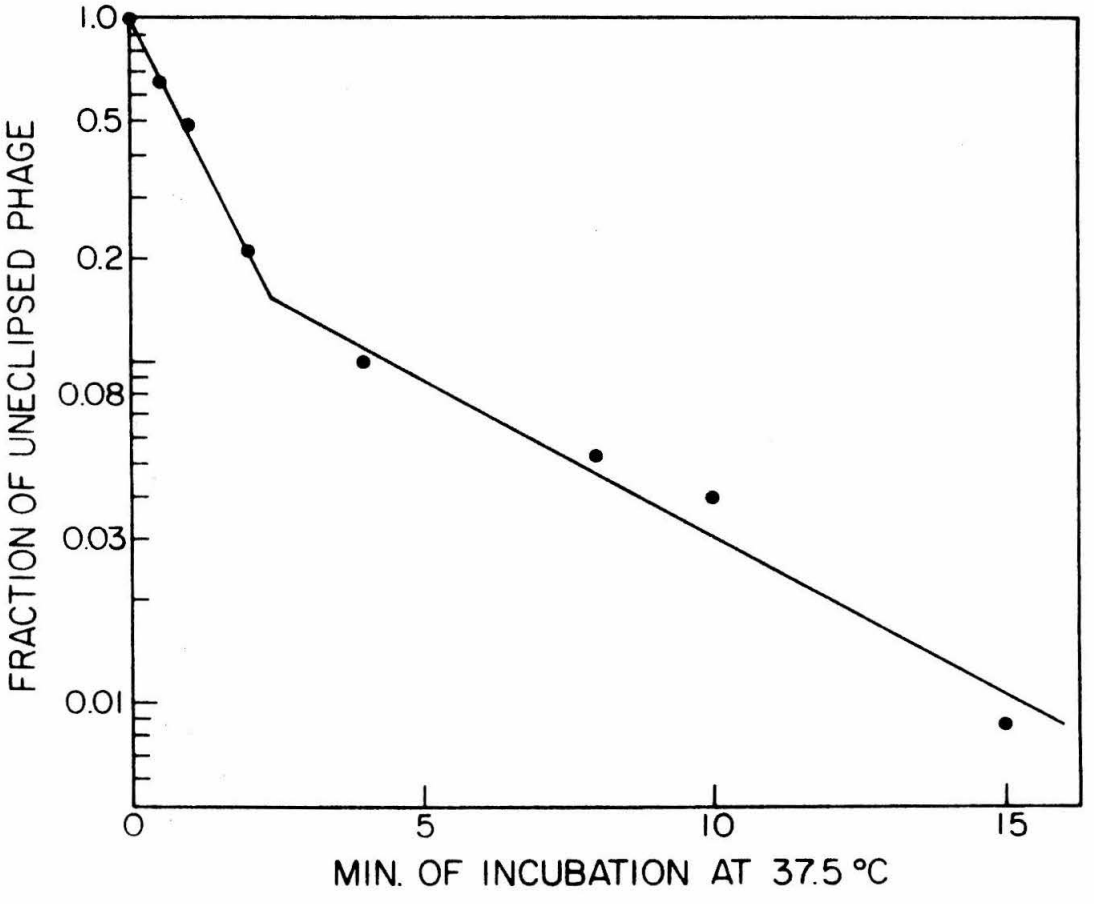
The product of the attachment rate constant and the electrophoretic mobility for some of the ØX strains. The electrophoresis data on whole phage are taken from reference (5).

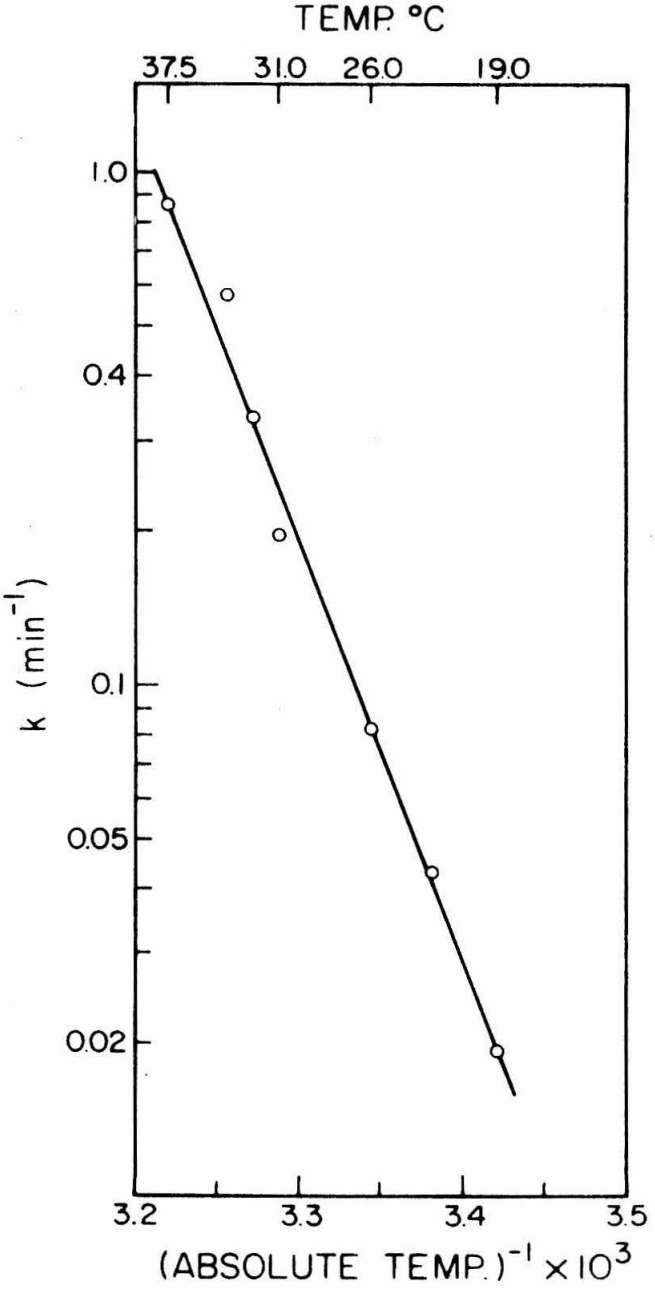
Phage strain	Rate constant for attachment, k $ml\text{-}min^{-1}\text{-}bact.^{-1} \times 10^{9}$	Electrophoretic mobility R_F	Product $k \times R_F$
wt	8.2	0.20	1.64
tsχ	8.5	0.20	1.70
tsχh	11.3	0.14	1.58
ts79	5.1	0.31	1.58

FIGURE LEGENDS

FIG. 1. The kinetics for the eclipse at 37.5°C of ØXam3 previously attached to E. coli C cells.

FIG. 2. The temperature dependence of ØX am3 eclipse. The Arrhenius plot of the logarithm of the initial rate constant for phage eclipse versus the inverse of the absolute temperature.


1.0
0.5
0.2
0.08
0.03
0.01
FRACTION OF UNECLIPSED PHAGE
0
5
10
15
MIN. OF INCUBATION AT 37.5 °C


TEMP. °C
37.5
31.0
26.0
19.0
1.0
0.4
0.1
0.05
0.02
k (min⁻¹)
3.2
3.3
3.4
3.5
(ABSOLUTE TEMP.)⁻¹ × 10³

II 3 Discussion

The first step in the invasion of the cell by ØX174 is the stable attachment of the phage to the receptor site on the host cell wall. The experiments described in section II. 2.1 indicate that the attachment step does not involve a detectable conformational change to the virion that is not both rapidly and completely reversible. Purified 114S virions attach efficiently to cells at 15°C and can be recovered as such, with full infectivity, upon application of the borate-EDTA elution procedure to the phage-cell complexes. However, no data were presented relevant to the conformation of the phage particle while it is attached to the cell. The electron microscope studies of E. coli C cells with ØX attached at extremely high multiplicities (550 PFU/cell) reveal the attached phage, by negative staining, identical in gross appearance to free virions similarly prepared (Stouthamer, Daems & Eigner, 1963, Daems, Eigner, Van der Sluys & Cohen, 1962).

Other investigations have implicated a possible conformational change in the virion prior to, and necessary for, stable attachment. Bleichrodt and Abkoude (1967) have described a partially reversible transition between a non-adsorbing, thermo-resistant form of ØX and the usual adsorbing, thermo-sensitive form. The two forms, denoted Ø* and Ø respectively, were found to be indistinguishable by sedimentation criteria. Application of the theory of absolute reaction rates

for the transition from the ϕ^* into the ϕ form suggests that the structural difference between the two forms may be determined by only one weak noncovalent bond (Bleichrodt, Blok & Abkoude, 1968). It seems likely that the attachment studies reported in this thesis relate to phage in the ϕ form and not to the ϕ^* form. The possibility that the ϕ^* form consists of virions in association with a small piece of bacterial debris has not been eliminated.

Fujimura (1961) described a conformational change experienced by ØX when incubated in 0.1M $CaCl_2$. The change was revealed by sedimentation analysis in the same salt solution -- a preparation of 114S virions was converted, partially, to 98S particles. The 98S species was able to attach to a cell wall preparation of E. coli C, while the 114S component did not. Investigations in this laboratory with ^{32}P, ^{3}H-L-leucine ØX am3 have also detected the conversion of 114S virions into the more slowly sedimenting (91S) species (Newbold, unpublished experiments). The ratio of ^{32}P to ^{3}H radioactivity for both species (114S and 91S) was the same as for the initial phage stock. The unconverted 114S species was also identical to the initial virus in its specific infectivity and capability for specific attachment to whole cells. The 91S particles, however, displayed a reduced capability for specific attachment to whole cells and only one tenth of the specific infectivity of the initial phage stock. Experiments with the micrococcal nuclease demonstrated that 45% of the DNA of the 91S particle could

be digested to an acid soluble form; the DNA of the 114S particles was resistant to digestion. Rather than being a necessary conformation for phage attachment, the 91S particle would seem to represent an early stage of the calcium-induced degradation of ØX (Guthrie & Sinsheimer, 1960).

Three ØX cistrons, viz II (spike protein), III (spike protein) and VII(capsid protein) are demonstrated to affect the attachment interaction. Two mutants, ts79 (cistron III) and h (cistron VII), which are both electrophoretic variants of the wild type, exhibit altered rates of attachment to E. coli C. The data suggest that the total charge of the virion is an important determinant of the rate at which stable attachment is effected. This, in turn, would further suggest that the proteins coded by cistrons III and VII need not be directly involved in the attachment interaction.

The ts4 mutant of cistron II produces in infection at the restrictive temperature a 110S defective particle (Krane, 1966). This particle contains an infectious SS DNA molecule, lacks a minor protein component of the phage spikes (Edgell, et al., 1969) and is unable to attach to cells. Conceivably, the inability to attach to cells may also be an indirect consequence of an altered charge or shape, but it is also possible that cistron II protein is a specific attachment organelle (at least when incorporated into a spike). If indeed cistron II does specify

an attachment protein, and the influence of other structural proteins on attachment is indirect, then the extended host range of the h mutant may also be determined by the total charge on the virion.

Following the stable attachment of the virus to the cell the attached phage, at normal temperatures, rapidly undergo an irreversible conformational change which defines their "eclipse". The altered conformation of the eclipsed phage may involve a partial penetration of the viral DNA into the cell. At the very least eclipse must involve a rearrangement of the protein subunits of the phage coat. The Arrhenius activation energy for the initial rate at which attached phage eclipse is 36.6 kcal/mole; this is a relatively high value and indicates that the phenomenon is cooperative in character.

Intimately associated with the conformational change that defines the eclipse event is the spontaneous detachment from the cell of a substantial fraction of the non-PFU in the phage-stock; those phage particles that remain attached to the cell are now more tightly bound to the cell than prior to the eclipse event. It is possible that the tighter binding of the attached phage particles upon eclipse reflects the participation of a larger number of the subunits of the phage coat in the direct interaction with the bacterial receptor site.

The normal eclipse phenomenon can be modified by viral mutation, but at present it is not possible to identify which ØX cistrons

are involved. The cold-sensitive mutant cs70 attaches apparently normally at 25°C, but does not eclipse at that temperature (Dowell, 1967); the wild type ØX does eclipse at 25°C. The mutant cs70 also exhibits an altered thermo-sensitivity and a more rapid rate of phage maturation. The cs70 mutant strain may represent a multiple mutant or a single mutant having a pleiotropic effect. However, the cold-sensitive lesion has not yet been assigned to a specific ØX cistron by genetic analysis; the nature of the lesion does not permit a complementation test and recombination studies have not yet been undertaken. The cs70 mutation was tentatively assigned to ØX cistron IV by analogy with other mutants that exhibit altered thermo-sensitivity (Dowell, 1967).

One protein that may play a role in eclipse is that specified by cistron III. The ØX mutant tss6, assigned to cistron III, displayed an increased level of spontaneous detachment associated with its eclipse in the restrictive infection (Dalgarno & Sinsheimer, 1968). Moreover, the kinetics with which tss6 completed the two-step process of stable attachment followed by eclipse were aberrant under the restrictive condition; either attachment or eclipse, or both, were implicated as impaired. Evidence was also presented which suggested that the tss6 mutation could give rise to abortive penetration of viral DNA (Dalgarno & Sinsheimer, 1968). ØX cistron III might be involved in all three of the early stages of infection, i.e. attachment, eclipse and DNA

penetration. However, it should be realized that the phenotype of defective DNA penetration could be the result of improper eclipse and that improper attachment might lead to defective eclipse, increased spontaneous detachment and abortive DNA penetration.

Analysis of ØX mutants of known cistron assignment for an altered initial rate of eclipse revealed no aberrant strains. Since the analysis included the mutants ts79 (cistron III) and h (cistron VII) that have an altered electrophoretic mobility (Hutchison, Edgell & Sinsheimer, 1967), charge may not play a role in the eclipse phenomenon.

As previously inferred by Rueckert and Zillig (1962) the kinetics for the eclipse of attached phage are biphasic; the majority of the phage eclipse at one rate and the remainder do so at a distinctly lesser rate. Rueckert and Zillig suggested that heterogeneity among the phage receptor sites on the host may be responsible for the phenomenon.

In the presence of nutrients, penetration of the viral DNA follows the eclipse event for those phage that are PFU and generates an empty phage protein coat that remains firmly attached to the exterior portions of the cell. The penetrated viral DNA is found in the cell as RF. That the host plays some role in this penetration event is evidenced by the inhibition of the event in the infection of starved cells maintained in the starvation medium (Knippers et al, 1969). An active cell metabolism does not seem to be the function provided by the host

since DNA penetration is not inhibited in infections in KCN or NaN_3. It has been suggested that some favorable condition of the cell wall or membrane that is affected by the starvation treatment is the limiting condition to the complete penetration of the phage DNA (Knippers et al., 1969). Since temporally excluded, superinfecting phages are blocked at the same stage of the invasion process, the same non-permissive condition of the cell wall or membrane may be induced by the primary infection.

Preliminary experiments concerning the interaction of ØX with isolated bacterial receptor — a lipopolysaccharide material — indicate that the in vitro eclipse reaction mimics the in vivo phenomenon. The data indicate that DNA penetration does not occur however, suggesting that host components other than the receptor site for attachment may be required (J. A. Cameron, personal communication).

Inhibition of host DNA synthesis prior to ØX infection does not prevent the separation of the viral DNA from the phage coat. The phage genome is found associated with the cell as a SS DNA molecule (Knippers et al, 1969). It has been suggested, although not demonstrated that this SS DNA molecule is within the bacterium and exposed to the bacterial cytoplasm; it was inferred that in the normal infection the viral DNA traversed the cell wall and membrane as a SS DNA molecule and was thereafter rapidly converted to RF. The penetrated

SS DNA is associated with the membrane fractions of gently-lysed cells (Heath, personal communication). It is not yet apparent from these experiments that the viral DNA does in fact penetrate as a SS DNA ring. The membrane-associated, penetrated SS DNA contains a high proportion of linear molecules which may arise by artifactual means. Since the ring form of ØX DNA is required for infectivity in the spheroplast assay (Fiers & Sinsheimer, 1962) it has been assumed that ØX DNA would penetrate cells as the ring form.

The infectivity of isolated ØX DNA also suggested that the penetration of other components of the virion were not essential for productive infection. The fate of the phage protein upon the penetration of the viral DNA is to form an empty phage protein coat which remains bound to the bacterial surface. The isolated phage coat is a stable structure which is similar to, but distinct from, ØX top component. However, the analysis could not exclude the possibility that a small amount of viral protein per PFU does penetrate the cell in the normal infection. Since such a structural component of the virion could conceivably be rematured into progeny phage particles experiments were undertaken to detect a transfer of label (^{3}H-L-histidine) in parental viral protein to progeny phage (Newbold, unpublished experiments). An association of a small amount of parental label with progeny phage was detected (corresponding to 10,000 to 30,000 daltons of protein

per PFU). Control experiments had intimated that the apparent transfer was not caused by an aggregation of parental phage, eclipsed phage or phage coats with progeny phage, or by degradation of parental protein to the amino acid level and reincorporation. Acrylamide gel electrophoresis of the "transferred" protein revealed it to be indistinguishable from the viral capsid protein (Mayol, unpublished experiments). However, if the other minor protein components present in whole phage had been present in this material in the proportions characteristic of the parental virion they would not have been detected. If indeed a transfer of parental viral protein occurs then the data would suggest that at most only one molecule of capsid protein (about 48,000 daltons) per PFU is transferred from the infecting phage to the progeny.

The invasion of E. coli C by bacteriophage ØX174 is another example of invasion by injection of viral DNA. ØX cistron II protein has been implicated as an analogue of the tail fibers of the T-even phages. Whether or not the other components of the phage spikes have their analogues too must await further study. Perhaps ØX is a symmetrical virion with 12 equivalent attachment organelles. At present there are no data to demand a contrary view. It is interesting to note, however, that if indeed the spherical RNA phages contain only one molecule per virion of an attachment (maturation) protein, as has been suggested (Roberts & Steitz, 1967), then they must be considered

asymmetric. It remains then an interesting problem to determine the equivalence or the non-equivalence of the ØX spikes.

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